STUDIES OF HSP90 FUNCTION ON SIGNAL TRANSDUCTION MOLECULES IN C2C12 CELLS AND EFFECTS OF NOVOBIOCIN ON HSP90 CONFORMATION AND FUNCTION AND MECHANISM OF REGULATION OF HEME-REGULATED eIF2α KINASE BY GAS MOLECULES

By

BO-GEON YUN

Bachelor of Science

Kangwon National University

Kangwon-do, Korea

1994

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STUDIES OF HSP90 FUNCTION ON SIGNAL TRANSDUCTION MOLECULES IN C2C12 CELLS AND EFFECTS OF NOVOBIOCIN ON HSP90 CONFORMATION AND FUNCTION AND MECHANISM OF REGULATION OF HEME-REGULATED eIF2α KINASE BY GAS MOLECULES

Thesis Approved:

L. Robert Matts

_____

Thesis Adviser

C. Richard Essenberg

_____

C. Eldon Nelson

_____

Andrew Mort

_____

L. Robert Burnap

_____

A. Gordon Emslie

_____

Dean of the Graduate College

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ABBREVIATIONS

ADP	adenosine triphosphate
ALLN	N-acetyl-Leu-Leu-norleucinal
ANPR	atrial natriuretic peptide receptor
APC	anaphase-promoting complex
ATP	adenosine triphosphate
cAMP	3', 5'-cyclophosphoric acid
Cdc	cell division cycle
Cdk	cyclin-dependent kinase
CFTR	cystic fibrosis transmembrane conductance regulator
CO	carbon monoxide
CREB	cyclic AMP response element binding protein
c-Src	cellular Src kinase
Cyp40	40-kDa cyclosporine A-binding protein
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DTT	dithiothretol
EDTA	ethylenediaminetetraacetic acid
eEF	eukaryotic elongation factor
EGTA	ethylene glycol-bis(2 aminoethyl)-N,N,N',N'-tetraacetic acid
eIF2α	α subunit of the eukaryotic initiation factor 2
ErbB2	Epidermal growth factor receptor subunit 2
Fak	focal adhesion kinase
FKBP	FK506 binding protein
GA	geldanamycin
Gcn2	general control non-derepressible-2
GSK3	glycogen synthase kinase-3
HA	hemagglutinin
Hck	hemopoietic cell kinase
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
Hop	Hsp70-Hsp90 organizing protein
HRI	heme-regulated inhibitor of protein synthesis
Hsc	heat shock cognate protein
Hsp	heat shock protein
IgG	Immunoglubulin G
JNK	c-Jun N-terminal kinase
Lck	lymphoid cell kinase
MEK	mitogen-activated protein kinase/extracellular signal-regulated kinase kinase
MKK	mitogen-activated protein kinase (MAPK) kinase

MOK	MAPK/MAK/MRK overlapping kinase
MRF	myogenic regulatory factor
Myf5	one of MRFs
NO	nitric oxide
NT-HBD	N-terminal heme-binding domain of HRI
HRI/ΔHBD	mutant HRI lacking NT-HBD
ΔH-HRI	mutant HRI lacking H-helix within NTD
NT-HBD/ΔH	NT-HBD lacking H-helix and linker region
Lck	lymphoid cell kinase
PARP	poly (ADP-ribose) polymerase
PDK	phosphoinositide-dependent protein kinase
PIPES	piperazine-N,N'-bis[2-ethanesulfonic acid]
PI3K	phosphatidylinositide-3-OH kinase
PKR	double-stranded RNA-activated protein kinase
PP1	phospho-protein phosphatase 1
PP2	phospho-protein phosphatase 2A
PVDF	polyvinylidene difluoride
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Sevenless RTK	sevenless receptor tyrosin kinase
Src	rous sarcoma virus oncogene
SHR (SR)	steroid hormone receptor
17-AAG	17-allylamino-17demethoxygeldanamycin

CHAPTER I

Literature Review: Molecular Chaperones; Regulation of signaling proteins in cells

Christian Antifsen and his colleagues concluded that "no special genetic information is required for the proper folding of the (protein) molecule and for the formation of 'correct' disulfide bonds" (1). The native conformation of a protein is postulated to be its most stable structure thermodynamically with the information specifying the structure coming from its primary amino acid sequences (1). A major driving force of folding non-native or intermediate polypeptides is the collapse of hydrophobic amino acid side chains away from an aqueous environment to form a hydrophobic molten globule core (2).

For over a decade, the area of protein folding research has focused on understanding the function of the cell's folding machinery in such processes as, the regulation of protein conformation and function, protein trafficking and degradation, and on the molecular basis of amyloid diseases [reviewed in (3-7)]. The cell's folding or chaperone machinery consists of several different conserved protein families and was classified originally by their approximate molecular weights and the environmental or physiological conditions that induced their synthesis. While previously referred to as heat shock proteins (Hsp) or glucose-regulated proteins (Grp), these proteins are now known to be constitutively expressed in cells and to play a critical role in guiding proteins to their correct conformations in cells (8).

However, macromolecules inside cells encounter a highly crowded environment that causes excluded volume effects, which can lead to increased aggregation of proteins (9). Cells appear to have evolved molecular chaperones to facilitate the folding of newly synthesized proteins, while protecting them from aggregation, and to rescue existing proteins that are partially denatured by stresses, such as heat shock and oxidative stress (10, 11). Molecular chaperones carry out their function to ensure that correct and efficient folding is achieved within the highly crowded environment of a living cell (6). Thus, the correct conformation of a protein is essential for its proper function and the maintenance of a cell's physiology, and defects in protein folding are now known to be factors in a number of human diseases, such as cystic fibrosis, cancer, and neurodegenerative diseases including Huntington's, Alzheimer's, and Parkinson's diseases, and Creutzfeldt-Jakob disease (12).

Several of the major molecular chaperone families have been well studied over the last decade. However, I will review the properties and functions of Hsp90 and its co-chaperones, as these proteins and their targets are the focus of the research described in my thesis. Components of some of the major Hsp90 co-chaperones are listed in Table 1.

Hsp90 (Heat Shock Protein 90)

Hsp90 is an abundant and ubiquitous 90 kDa protein [reviewed in (13-15)]. It is a highly conserved chaperone that is required for folding and regulation of numerous signal transduction molecules, such as steroid hormone receptors, tyrosine and serine/threonine protein kinases, and other proteins like nitric oxide synthase and telomerase (13-15). Hsp90 is known to be critical for viability of eukaryotic cells and organisms, such as

Caenorhabditis elegans, *Drosophila*, and yeast (13-15). While Hsp90 is dispensable for viability in *Escherichia. coli*, it is essential for thermotolerance in cyanobacteria (13-15).

Members of the Hsp90 family are expressed and function in specific subcellular compartments. There are two nuclear/cytoplasmic isoforms, α and β, of Hsp90 in vertebrate cells, but there are apparently four isoforms of Hsp90 in *Arabidopsis thaliana*. There is also an isoform of Hsp90 targeted to the endoplasmic reticulum (Grp94: 94 kDa glucose-regulated protein) (16), and the mitochondria (TRAP1, type 1 tumor necrosis factor receptor-associated protein; or Hsp75) (17, 18). However, while the functions of Hsp90 and Grp94 have been investigated, its function in other organelles is not well understood.

Structure and function of Hsp90

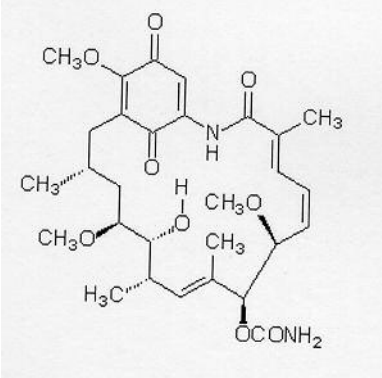
Both biochemical and crystallographic studies indicate that the N-terminus of Hsp90 contains a nucleotide-binding pocket and is the most conserved region of Hsp90 [reviewed in (14, 19)]. The N-terminus of Hsp90 binds to the inhibitors, geldanamycin (GA) (Fig. 1A and Fig. 3B) and radicicol. Unrelated in function to Hsp90, bacterial DNA gyrase (20), the DNA repair protein MutL (21), and histidine kinases (GHKL family) also share four common motifs with Hsp90 for binding ATP called the "Bergerat fold" (22, 23). All require ATP binding and hydrolysis to carry out their function which requires dynamic conformational changes. The binding and hydrolysis of ATP by Hsp90 is proposed to drive the opening and closing of a 'molecular clamp' (24). The jaws of the clamp are the N-terminal domains within the Hsp90 dimer, which associate upon binding ATP, and thus promote closing of the clamp (24) (See Fig. 2. model of Hsp90 ATPase cycle).

Recent studies suggest that the C-terminal region of Hsp90, which contains sequences responsible for Hsp90 dimer formation, has a second nucleotide-binding site (25). Binding of the drug, novobiocin (26) (Fig. 1B), to the C-terminal site blocks binding of ATP to Hsp90's N-terminal nucleotide binding site (27). Thus, regulation of Hsp90 function may be coordinated by the two nucleotide-binding sites undergoing dynamic conformational changes, which regulate nucleotide binding at the other site. However, the specific function of the second nucleotide-binding site of Hsp90 remains uncharacterized. Our recent study indicates that binding of nucleotide to the C-terminal site causes conformational changes in Hsp90, which may lead to client release (28).

The very C-terminus of Hsp90 contains a highly conserved MEEVD motif to which Hsp90 co-chaperones containing tetratricopeptide repeats (TPR), such as the immunophilins (FKBP52, FKBP51, cyclophilin-40/Cpr6/Cpr7), Hop/Sti1, and PP5, bind [reviewed in (29)] (See Fig. 3C). These TPR containing co-chaperones may regulate Hsp90 ATPase activity. Prodromou and co-workers (30) showed that Sti1 inhibits Hsp90 ATPase activity by binding to Hsp90 and blocking access to the ATP-binding site in the N-terminal domain of Hsp90. On the other hand, Cpr6 can reactivate Hsp90 ATPase activity by displacing Sti1 from Hsp90 (30).

The middle domain of Hsp90 is thought to contain one of Hsp90's client binding sites (31). A charged linker region connects the N- and the middle domain of Hsp90 (14) (Fig. 3A). This charged linker region is the least conserved between bacterial and eukaryotic Hsp90, and may play a key role in regulating chaperone function of Hsp90 by enhancing affinity of HSP90 for substrate and modulating ATP binding to the N-terminal

A. Geldanamycin



B. Novobiocin

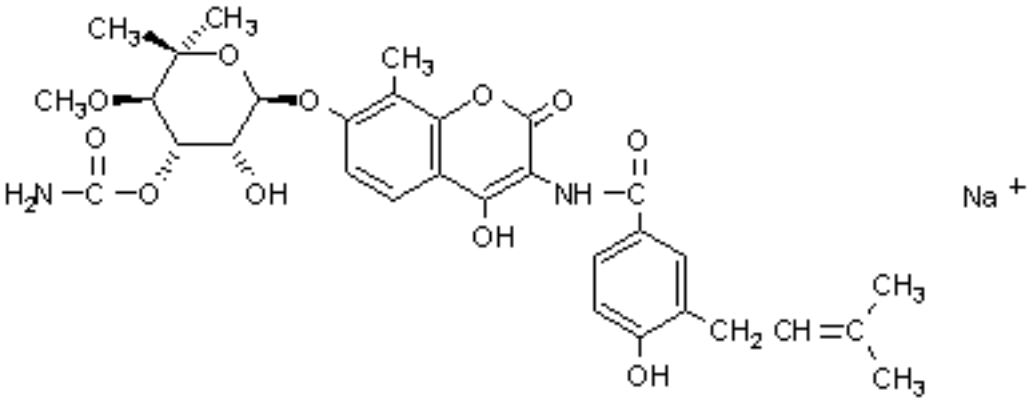


Figure 1. Structures of Geldanamycin (A) and Novobiocin (B).

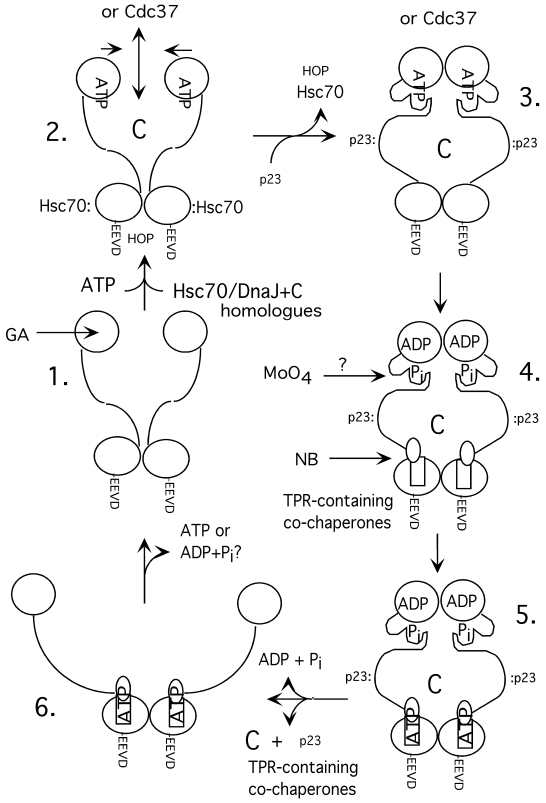


Figure 2. Model of Hsp90 ATPase cycle. 1.) Hsp90 in its default conformation with its N- and C-terminal ATP binding sites empty (speculative). 2.) Hsp90 containing ATP bound to its N-terminal domain and weakly bound client (C) after Hsc70/ATP-dependent loading of client. 3.) The conformation of Hsp90 formed following ATP- and p23-dependent clamping of Hsp90's N-terminal domain about bound client molecule (C). 4.) The high-affinity client bound conformation of Hsp90 formed following p23-stimulated ATP hydrolysis, which results in the opening of the C-terminal ATP binding domain (NB, arrow). It is currently postulated (? by arrow) that MoO_4^- stabilizes this conformation of Hsp90 by occupying the binding site of the γ-phosphate hydrolyzed from the ATP bound to the N-terminal domain of Hsp90. It is also uncertain a what step the γ-phosphate hydrolyzed from the ATP bound to the N-terminal domain is released. 5.) Hsp90 containing ATP (or novobiocin) bound to its C-terminal nucleotide binding site. 6.) Conformation of Hsp90 formed after dissociation of nucleotide from its N-terminal domain, which is induced by saturation of its C-terminal nucleotide binding site with ATP (or novobiocin). Novobiocin would arrest Hsp90 in this state, accounting for the absence of the association of p23 and Hsc70 with Hsp90 in the presence of novobiocin. 1.) Regeneration of the default conformation of Hsp90 due to the dissociation of ATP from its C-terminal nucleotide-binding site. It is uncertain whether the ATP bound to the C-terminal site is hydrolyzed (ATP or ADP+P_i?). The presence of Cdc37 and TPR-containing co-chaperones are indicated, but exact times and sites of interaction are generally, but not specifically, indicated to simplify the model. The binding of Cdc37 and Hop to Hsp90 appears to be mutually exclusive (or Cdc37).

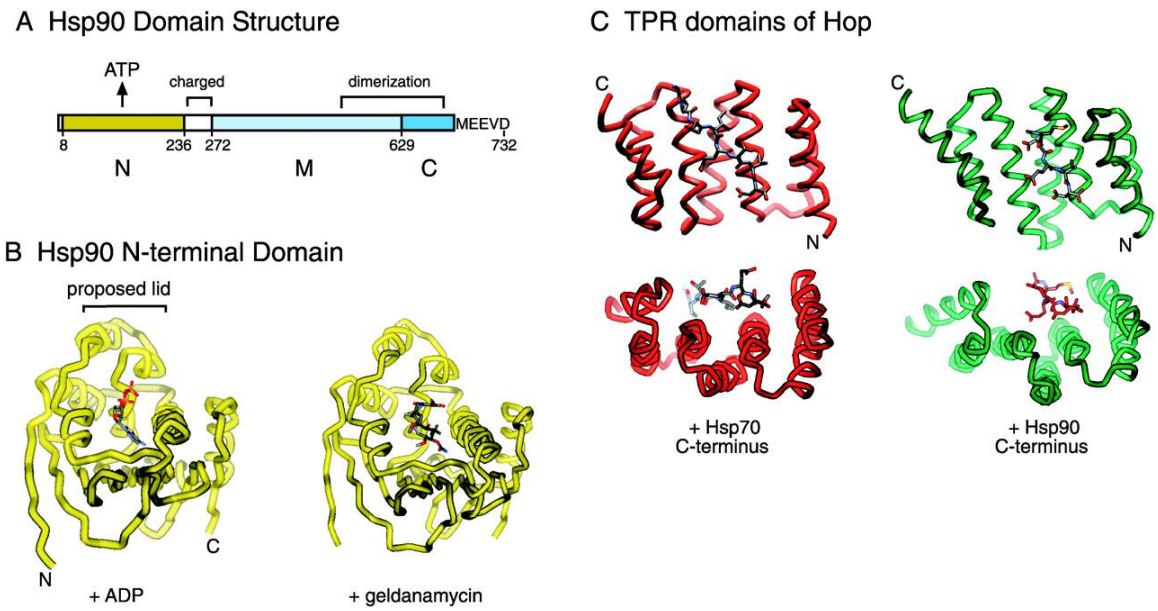


Figure 3. Structural basis for the interaction of Hsp90 with nucleotides, ansamycin drugs, and TPR domain co-chaperones. (A) Domain organization of human Hsp90α. (B) Crystal structure of N-terminal domain of Hsp90 complexed with ADP and geldanamycin. (C) Crystal structure of the Hsp70-binding N-terminal TPR domain of Hop complexed with the Hsp70 C-terminal peptide (GPTIEEVD) (left) and the Hsp90 C-terminal peptide (MEEVD) (right). Structures in the bottom panel are rotated 90° from the top panel. The peptides are oriented with the C-terminal carboxyl group at the bottom of the figure (top) and toward the viewer (bottom) [From Young et al. (14)].

region (32). However, a recent study suggests that both dimerization of the N-terminus and the association of the N-terminus with the middle domain are important for the efficiency of the ATPase cycle (33). This hypothesis is supported by recent finding that Hsp90 co-chaperone AHA1 binds to middle domain of Hsp90 and enhances Hsp90's ATPase activity (34).

Therefore, Hsp90's function as a molecular chaperone seems to require all three of its domains, which interact to modulate the conformation of Hsp90 in response to ATP binding and hydrolysis. Furthermore, Hsp90's co-chaperones are also thought to modulate Hsp90 chaperone activity in response to stimuli.

Hsp90 co-chaperones

As indicated earlier, Hsp90 functions in conjunction with cofactors that are not client targets. These so called co-chaperones are listed in Table 1. Here I will briefly explore the properties of these co-chaperones, and the current studies regarding possible regulation of Hsp90 by co-chaperones.

Cdc37 (p50)

Cdc37 was first identified as a 58 kDa protein product of a yeast gene, temperature-sensitive mutants of which arrested the cell division cycle at the nonpermissive temperature [reviewed in (35)]. Cdc37 is an essential protein known to be required for viability in yeast (36), *Drosophila* (37), and *Caenorhabditis elegans* (38).

Recently, Cdc37 was discovered to be an Hsp90 co-chaperone that is required for the proper folding and function of Hsp90-dependent protein kinases, such as Raf-1 (39),

Table 1

Cohorts/Co-chaperones	Properties
P23 Sba1 (yeast)	Stabilizes protein-Hsp90 heterocomplex ATP-dependent interaction
Hsp organizing proteins Hop Sti1 (yeast) Dpit47	Binds to form Hsp90-Hop/Sti1-Hsp70-client complex, prior to transfer of client to Hsp90 heterocomplex Hsp90-Dpit47 complex bound to DNA polymerase α
Immunophilins FKBP52 FKBP51 CyP40 Cpr6, Cpr7 (yeast) Cns1 (yeast)	 Bind to Steroid hormone receptor (SR)-Hsp90 heterocomplex Binds to dynein GR and v-Src signaling adversely affected by cpr7 null mutation, cns1 is essential for viability
Cdc37 (p50) Harc (Hsp90-associating relative of cdc37)	Bound to Hsp90-protein kinase complexes Binds Hsp90 directly A cytoplasmic phosphoprotein
PP5	Binds Hsp90 via TPR, found in SR-Hsp90 complexes and dynein
CHIP (C-terminus of Hsc70 interaction protein)	Interaction with Hsc70 causes proteasome-dependent degradation of substrate, binding to Hsp90 causes p23 release
UNC-45	Binds via N-terminal TPR to Hsp90 and via C-terminal regions to myosin
ARA9(called XAP2 and AIP)	Found in aryl hydrocarbon receptor (AHR)-Hsp90 heterocomplexes

Hsp90 binding cohorts/co-chaperones. [adapted from Pratt et al. 2003 (13)]

Cdk4 (40), and HRI (41). Cdc37 is composed of three domains: an N-terminal kinase binding domain; a middle Hsp90-binding domain; and a C-terminal domain of unknown function (42). Although its precise role in regulating Hsp90 function still needs to be fully characterized, the binding of Cdc37 to Hsp90 inhibits Hsp90's ATPase activity (43). Structural studies indicate that Cdc37 binds to the N-terminal domain of Hsp90 in its "relaxed" conformation, with the lid of the nucleotide-binding site open (44). This is thought to be the conformation of Hsp90 that required for the binding of client targets. However, Cdc37 also interacts with Hsp90 in complexes containing stably bound kinase clients (41). A C-terminally truncated Cdc37 mutant, which does not interact with Hsp90, stably binds kinase clients, and can inhibit their Hsp90-dependent folding (41). However, the binding of client protein to Hsp90-Cdc37 stabilizes the Hsp90-Cdc37 complexes (45). Thus, it remains to be determined whether Cdc37 modulates the stability and activity of the protein kinase clients by docking them to Hsp90, or by having a direct action on the client molecules.

Cdc37 may also have a chaperone function independent of Hsp90. Cdc37 can stabilize denatured casein kinase II (CKII), maintaining it in a state competent for refolding by other chaperones (46). Overexpression of Cdc37 in yeast can suppress the effects of Hsp90-deficiency (46). In addition, a recent study indicated that mutant yeast containing deletion of Cdc37's Hsp90 binding domain were viable under normal growth conditions (47). Furthermore, not all endogenous Cdc37 is associated with Hsp90.

Recent studies have demonstrated that nucleotide-mediated conformational switching of Hsp90 regulates the capacity of Cdc37 to bind to protein kinases (41). The Hsp90-inhibitor geldanamycin inhibits the ability of Cdc37 to bind the Hsp90-dependent

kinases, HRI and Lck, even though Cdc37 contains an independent protein kinase binding domain at its N-terminus (45). However, in the case of protein kinase B (Akt) geldanamycin does not appear to disrupt the binding of Cdc37 to Akt, but it does shorten the half-life of the kinase (48). Thus, Cdc37 appears to allosterically modulate the binding of Hsp90 to kinases, and acts in concert with Hsp90 to facilitate the folding and stabilization of protein kinases (42).

Co-chaperones containing tetratricopeptide repeat (TPR) domains

A number of Hsp90 co-chaperones, such as the immunophilins (FKBP51 and 52, and Cyclophilin 40), Hop (p60), and PP5, possess TPR domains. TPR domains are identified as degenerate consensus sequences of 34 amino acids that often are arranged in tandem repeat (49). These TPR domains are protein-protein interaction sites that are utilized to bind to the C-terminus of Hsp90, and possibly to client targets (49).

Immunophilins

The immunophilins are so named because they contain domains that bind to immunosuppressant drugs, such as FK506, rapamycin, and cyclosporin A. These domains have peptidylprolyl isomerase (PPIase) activity, suggesting they could have chaperone function. This family of co-chaperones is classified as two families: i) the FKBPs bind FK506 and rapamycin [as referenced by (50)], and ii) the cyclophilins (Cyps) bind cyclosporin A [as referenced by (50)]. These immunophilins contain additional TPR domains and FKBP52 contains a calmodulin-binding domain in its C-terminal region (51, 52).

FKBP52 was first identified as a component of Hsp90 complexes formed with steroid hormone receptors (SHR) [reviewed in (13, 50)]. Later, it was proposed that it

has a role in translocation of receptors to the nucleus (53, 54). Expression of human FKBP52 in yeast, which lack genes coding for this family of proteins, increased glucocorticoid (GR) hormone-binding affinity and its ability to transactivate reporter genes, while expression of FKBP51, PP5, or Cpr7 (yeast homolog of Cyp40) did not (55).

Cytoplasmic FKBP52 localizes to microtubules and associates with dynein via its PPIase domain (56). Binding of hormone has been shown to cause the exchange of FKBP51 for FKBP52, leading to the hypothesis that differential binding of TPR proteins to SHR complexes might be a switch regulating the subcellular trafficking of receptors (57).

Immunophilins may also have effects on the chaperone activity of Hsp90 that is required for SHR maturation and transactivation. In vitro, FKBP52 modulates the ATPase activity of Hsp90 (30). In yeast, Cpr6 (a homolog of Cyp40R) stimulates the ATPase activity by displacing Sti1 from Hsp90 (30).

Protein Phosphatase 5(PP5)

PP5 is a novel member of the serine/threonine (Ser/Thr) phosphoprotein phosphatase family that is expressed ubiquitously in mammalian cells (58). PP5 is a 58 kDa protein that is unique in that it posses three TPR motifs at its N-terminus (59). Polyunsaturated fatty acids, such as arachidonic acid, stimulate purified PP5 activity via disrupting the binding of its TPR domain to its catalytic domain, relieving its autoinhibition (60). PP5 also associates with Hsp90 via its TPR domain (61). PP5 has been identified in Hsp90 heterocomplexes containing SHRs (62), and HRI (21), and it

has been found bound to the atrial natriuretic peptide receptor, Cdc16p, and Cdc27p even in the absence of Hsp90 (63, 64).

Recent studies suggest that PP5 itself can interact and regulate apoptosis signal-regulation kinase-1 (ASK1) (65). PP5 appears to negatively regulate the hypoxia-induced ASK1/MKK4/JNK signaling cascade that promotes an apoptotic response (65). Interestingly, the C-terminal domain of Hsp90 stimulates the activity of PP5 in vitro (66). However, there is no evidence so far, indicating that PP5 may act to regulate Hsp90 function.

Hop (Hsp70-Hsp90 organizing protein, p60)

Hop was identified as a 60-kDa protein that interacted with complexes containing both Hsp90 and Hsp70 immunopurified from chick oviduct (as referenced by (50)). The yeast homolog of Hop is the nonessential stress protein, Sti1 (67). Hop is required for the assembly of 'early' SHR-Hsp90 heterocomplexes that first contain Hsp70, and subsequently acts as an adaptor to link the SHR-Hsp70 complex to Hsp90 (68). Hop possesses three distinct TPR domains, TPR1, TPR2A and TPR2B that allows Hop to bind both Hsp90 (via TPR2A) and Hsp70 (via TPR1), simultaneously (69, 70) [see Fig. 3C]. Hop preferentially forms a complex with ADP-bound form of Hsp70, but does not affect Hsp70's ATPase activity (71). On the other hand, Hop binds to ADP-bound Hsp90, and inhibits Hsp90's ATPase activity in vitro (71). Thus, Hop can modulate the function of Hsp90 by blocking the ATP dependent conversion of Hsp90 to a form capable of interacting with p23 (72). Binding of Sti1 to Hsp90 can displace geldanamycin (GA) from Hsp90's N-terminal nucleotide binding domain. In vitro, Hop interacts with both

the N- and C- terminal domains of Hsp90, and inhibits the N-terminal dimerization of Hsp90 that is required for efficient hydrolysis of ATP (73).

p23

p23 was identified as a 23-kDa protein associated with the avian progesterone receptor (PR) (74) and the murine glucocorticoid receptor (GR) (75). p23 has been shown to have passive chaperone activity, as it can prevent the aggregation of denatured protein *in vitro* [reviewed in(76)].

The stable binding of p23 to Hsp90 requires hydrolysable ATP, suggesting that p23 function is coupled to the ATPase activity of Hsp90 (77). Recently, p23 has been proposed to function in the release of Hsp90-bound client proteins (78). Studies utilizing purified factors have demonstrated that p23, in conjunction with Hsp90, Hsp70, Hsp40 and Hop, is required for the assembly of steroid receptor-Hsp90 heterocomplexes, and functional SHRs (79). However, genetic studies in yeast indicate that while p23 enhances Hsp90's function, it is not essential for activation of Hsp90-dependent clients (80). A recent study on p23 function has suggested that p23 may localize to the chromatin of hormone-responsive genes and facilitate the disassembly of transcriptional regulatory complexes (81).

Regulation of signal transduction pathways by Hsp90

Hsp90 activity has been demonstrated to be required for the proper folding and function of numerous signal transduction molecules in cells. A summary of known Hsp90 clients is shown in Table 2. This review will focus on Hsp90-dependent

Table 2

Classes of Substrates	Hsp90 substrates
Transcription Factors	Glucocorticoid receptor Progesterone receptor Estrogen receptor Androgen receptor Dioxin receptor p53, **MyoD**1, Heat shock factor Mineralcorticoid receptor Hypoxia-inducible factor-1α
Tyrosine Kinases	v-Src, **c-Src**, **Fyn** v-Fps, v-Yes, v-Fes, Frg, Hck, Lck **ErbB2**, Senvenless RTK, $p210^{bcr\text{-}abl}$ Wee1, Insulin receptor, Fak
Serine/Threonine Kinases	**HRI**, Gcn2, PKR eEF-2 kinase MEK, MOK, c-MOS **Akt (PKB),** PDK1, PI3K Raf kinases, Casein kinase II CDK4, CDK6, CDK9
Others	Nitric oxide synthases (NOS): Endothelial, Neuronal, & inducible. CFTR, ANPR, Apaf-1 Telomerase Hepatitis B virus reverse transcriptase

Identified Hsp90 clients. Bold Letter: Tested in this study.
Not all shown in this table adapted from (13, 82).

regulation of protein kinases determined primarily through the pharmacological effects of Hsp90 inhibitors on their function and stability. Many signal transduction molecules involved in cancer progression require Hsp90 function for their maturation and/or stabilization. These proteins include receptor tyrosine kinases, steroid hormone receptors, Src-family kinases, serine/threonine kinases, transcription factors, and cell cycle regulators, as summarized in Table 2. Obviously,
Hsp90 function is essential for cell proliferation and survival [reviewed in (10, 12-14)]. However, the function of many critical signal transduction molecules is altered by mutations, and in many cases the stability of the altered gene products have an exaggerated requirement for Hsp90. Thus, Hsp90 function becomes essential for these mutant proteins to cause the imbalance of signal transduction pathways that leads to the oncogenic transformation of normal cells.

For example, the receptor tyrosine kinase ErbB2 (also called Her-2/Neu) plays key roles in regulating cell proliferation [reviewed in (83)]. Recent studies have shown that overexpression or mutation of ErbB2 can cause malignancies, including ovarian cancer, cell carcinoma of the head and neck, glioblastoma, lung cancer, and breast cancer (84, 85). ErbB2 requires Hsp90 function which is critical for its maturation, stabilization and regulation in signal transduction pathways (86, 87). Inhibition of Hsp90 function by geldanamycin (GA) or its derivative, 17allylamino-17-demethoxygeldanamycin (17-AAG), destabilizes ErbB2 and induces proteasomal degradation of ErbB2 through its polyubiquitination and thus reduces tumor cell growth (86, 88).

The Ras/Raf/Mek/MAPK signaling pathway regulates cell proliferation and survival by transducing cell signals from the cell surface to the transcriptional machinery

(89). Dysregulation (constitutive activation) of Ras/Raf signal transduction pathways causes oncogenic transformation of cells, and has been identified to occur in various cancers (90). Geldanamycin (GA) disrupts Raf-1-Hsp90 complexes, causes destabilization of Raf-1 and loss of Ras-Raf-1 association, which results in inhibition of Raf-1/Mek/MAPK signaling pathway (91, 92).

PI3K/PDK/Akt signaling pathway plays central roles in cell proliferation, growth, and survival [reviewed in (93-96)]. Phosphatidylinositide-3-OH kinase (PI3K) and 3-phosphoinositide-dependent kinase-1 (PDK1) are upstream signaling molecules of Akt. Akt regulates the phosphorylation of downstream effectors, such as GSK-3, Bad, Forkhead, NF-κB, mTOR, and MDM-2, that have been demonstrated to contribute to angiogenesis, cancers of the prostate and breast, and the hypertrophy of muscle cells [reviewed in (97)]. These signaling molecules are known Hsp90 clients. Inhibition of Hsp90 function by geldanamycin (GA) leads to destabilization of these molecules and disruption of these signaling pathways (98). A recent study suggests that inhibition of Hsp90 binding to Akt, stimulates Akt dephosphorylation via PP2A, leading to the suppression of Akt's kinase activity (99). In ErbB2 overexpressing cancer cells, 17-AAG also stimulates Akt dephosphorylation prior to loss of Akt expression (98).

A recent study on Hsp90 inhibition by 17-AAG reported that Hsp90 derived from tumor cells has a 100-fold higher binding affinity for 17-AAG than does Hsp90 from normal cells (100). They postulate that this higher binding affinity is due to Hsp90 being present in multi-chaperone client complexes with high ATPase activity in tumor cells, while in normal cells most of the cell's Hsp90 appears to be in client free complexes (100). This finding suggests an explanation for the observation that Hsp90 inhibitors are

selectively toxic to tumors cells, and why Hsp90 inhibitors appear to be efficient drugs for cancer treatment.

My studies presented in the subsequent chapters will mainly focus on the function of Hsp90, using Hsp90 inhibitors, in particular novobiocin, to alter to Hsp90's conformation, function and interaction with clients in reticulocyte lysate. Additional studies examine the effect of Hsp90 inhibitors on signal transduction molecules and pathways in differentiating C2C12 myoblasts.

CHAPTER II

Novobiocin induces a distinct conformation of Hsp90 and alters Hsp90-cochaperone-client interactions

Abstract

Hsp90 functions to facilitate the folding of newly synthesized and denatured proteins. Hsp90 function is modulated through its interactions with co-chaperones and the binding and hydrolysis of ATP. Recently, novobiocin has been shown to bind to a second nucleotide binding site located within the C-terminal domain of Hsp90. In this report, we have examined the effect of novobiocin on Hsp90 function in reticulocyte lysate. Novobiocin specifically inhibited the maturation of the heme-regulated eIF2α kinase (HRI) in a concentration dependent manner. Novobiocin induced the dissociation of Hsp90 and Cdc37 from immature HRI, while the Hsp90 co-chaperones p23, FKBP52 and protein phosphatase 5 remained associated with immature HRI. Proteolytic fingerprinting of Hsp90 indicated that novobiocin had a distinct effect on the conformation of Hsp90, and molybdate lowered the concentration of novobiocin required to alter Hsp90's conformation by ten fold. Recombinant C-terminal domain of Hsp90 adopted a proteolytic resistant conformation in the presence of novobiocin, indicating that alteration of Hsp90/co-chaperone interactions was not the cause of the novobiocin-induced protease resistance within Hsp90's C-terminal domain. The concentration dependence of this novobiocin-induced conformation change correlated with the

dissociation of Hsp90 and Cdc37 from immature HRI and novobiocin-induced inhibition of Hsp90/Cdc37-dependent activation of HRI's autokinase activity. The data suggest that binding of novobiocin to the C-terminal nucleotide-binding site of Hsp90 induces a change in Hsp90's conformation leading to the dissociation of bound kinase. The unique structure and properties of novobocin-bound Hsp90 suggests that it may represent the "client-release" conformation of the Hsp90 machine.

Introduction

Numerous proteins that are involved in the control of physiological processes within cells require the Hsp90 chaperone machine for their biogenesis and regulation [reviewed in (13, 82, 101, 102)]. While Hsp90 can function to facilitate the renaturation of misfolded proteins (103-107), it is not required for the biogenesis of most proteins synthesized in a cell. Rather, Hsp90's specific clientele is primarily restricted to certain proteins involved in the regulation of signal transduction [reviewed in (13, 82, 101, 102)]. Additionally, Hsp90 supports mutational drift in proteins (108, 109) that may be essential to the microevolution of transformed cell populations. Thus, Hsp90's essential function as a chaperone, which is required for regulation of cellular signal transduction makes it an attractive target for efforts to manipulate the growth and differentiation of normal and aberrant cell populations [reviewed in (8, 110, 111)].

Hsp90 functions through its interactions with numerous co-chaperone partners [reviewed in (13, 82, 101, 102)]. Some of Hsp90's co-chaperone partners are thought to modulate the interaction of Hsp90 with specific families of clientele. For example, Hsp90's co-chaperone Cdc37 is required for the biogenesis of numerous Hsp90-dependent protein kinases [reviewed in (35)]. Besides mediating the binding of Hsp90 to

specific client targets, Hsp90-associated co-chaperones also modulate Hsp90's ATP-driven reaction cycle and Hsp90's nucleotide–modulated conformation switching.

Hsp90 binds and hydrolyzes ATP via a Bergerat fold (112, 113) within its N-terminal domain, which mediates global switching between at least two alternative Hsp90 conformations [reviewed in (13, 82, 101, 102)]. The binding of ATP to Hsp90's N-terminus induces the N-terminal domains within the Hsp90 dimer to associate, forming a "molecular clamp" about its client target (24, 33, 114-116). The Hsp90-specific inhibitor geldanamycin binds to this nucleotide-binding site and prevents the formation of the closed conformation of Hsp90 (117, 118), resulting in the destabilization of Hsp90-kinase complexes. Geldanamycin, thus causes the accumulation of "intermediate" Hsp90 complexes (119, 120) containing Hsc70 and the Hsp70-Hsp90 organizing protein p60/HOP (a homolog of the yeast protein, Sti1), as it prevents the progression of Hsp90 through its ATP driven reaction cycle. Sti1 appears to repress Hsp90's ability to consume ATP (30, 73, 121), as does Cdc37 (43, 44).

The C-terminal region of Hsp90 mediates Hsp90's stable dimerization (33, 116, 122, 123) and contains a second nucleotide-binding site (26, 27, 124). Novobiocin binds to this nucleotide-binding site and inhibits Hsp90 function (25-27). The C-terminal region together with Hsp90's middle domain mediates the interactions of Hsp90 with molybdate (27, 125), protein clients (126, 127) and modulates Hsp90's ATPase activity (33, 128). Binding of ATP to Hsp90's N-terminal domain is required for the second ATP site to become available for the binding of nucleotide (26, 27, 124), and for the ability of molybdate to stabilizes Hsp90-client complexes (77). The ability of molybdate binding to "freeze" Hsp90's ATPase cycle causes the accumulation of "late" Hsp90 complexes

containing the Hsp90 co-chaperone p23 and an assortment of other co-chaperones, such as Cdc37 and immunophilins (41, 77, 125, 129, 130). Mutagenesis studies indicate that formation of stable complexes between p23, Hsp90 and client requires the N-terminal domain of Hsp90, the presence of hydrolysable ATP, and sequences within the C-terminal domain of Hsp90 (24, 77, 114, 118, 128, 131-133). The binding of p23 has also been proposed to stimulate the release of client substrate (78), and novobiocin appears to disrupt the interaction of p23 (26, 134) and Hsp70 (26) with Hsp90. Thus, Hsp90 function is regulated by a complex series of reactions that are modulated by the binding, hydrolysis and presumably exchange of ATP at two sites, and the interactions of Hsp90 with co-chaperones and client target proteins.

While reports indicate that novobiocin treatment of cultured cells leads to the loss of Hsp90-dependent protein kinases (25), mutant p53 (25) and HIF-1α (135), and that novobiocin disrupts the binding of Hsp90 and Hsp70 to the glucocorticoid receptor *in vitro* (136), and the AKT kinase in cells (137), the effect of novobiocin on the interaction of Hsp90 with other clients and Hsp90-associated co-chaperones has yet to be studied in detail. In this report, we have examined the effect of novobiocin on Hsp90-dependent maturation of the heme-regulated eIF2α kinase (HRI), Hsp90's conformational switching and its interactions with other co-chaperone partners and HRI under quasi-physiological conditions in rabbit reticulocyte lysate. The data indicate that novobiocin inhibits Hsp90 function by inhibiting Hsp90's ATP-driven chaperone cycle at a distinct stage. The properties and structure of novobiocin-bound Hsp90 distinguish this Hsp90 conformation from those it adopts when it is bound to geldanamycin or molybdate.

Experimental Procedures

Analysis of the effects of novobiocin on the maturation and activation of HRI in rabbit reticulocyte lysate. Untreated reticulocyte lysate was prepared by injecting New Zealand White rabbits with 1 mg/Kg of 1% N-acetylphenylhydrazine (w/v) in sterile water for 5 days. After 4 days of recovery, blood was collected and washed four times with buffer containing 10 mM Hepes-KOH (pH 7.2), 134 mM NaCl, 5 mM KCl, 7.4 mM magnesium acetate and 5 mM glucose. Reticulocytes were lysed in one volume of sterile deionized water, and lysate collected after centrifugation at 20,000 x g for 10 min. Normal untreated reticulocyte lysate used in maturational incubations was incubated under conditions for protein synthesis with or without the addition of 20 mM hemin as described previously (138).

[^{35}S]-His-tagged HRI was synthesized for 30 min at 30 ºC by coupled transcription/translation in nuclease-treated rabbit reticulocyte lysate (TnT, Promega) as previously described (41, 129, 138-140). Following inhibition of initiation by the addition of aurintricarboxylic acid (60 µM final), HRI was then incubated with drug for the times indicated in the figures, or aliquots (3 µl) of the reaction mixtures were transferred to untreated heme-deficient rabbit reticulocyte lysate protein synthesis mixtures (22 µl) containing drug or vehicle control. Reaction mixtures were then incubated at 30 ºC for 65 min to determine the effects of the drugs on the maturation and activation ("transformation") of HRI.

Immunoadsorption of complexes of HRI and components of the Hsp90 chaperone machine from rabbit reticulocyte lysate. [^{35}S]-His-tagged HRI was synthesized as described above, then mixed with seven volumes of heme-deficient rabbit reticulocyte

lysate that was pretreated with drug or vehicle control for 5 min at 30 ºC, and further incubated for 60 min at 30 ºC. Co-adsorption of Hsp90 and Cdc37 in chaperone-HRI heterocomplexes was analyzed by adsorption with anti-(His_5) antibody (Qiagen) as described previously (41, 129, 138). To analyze the effect of novobiocin on the interaction of HRI with other co-chaperones, HRI was synthesized and matured in the presence or absence of drug as described above. Reaction mixtures were divided in half and incubated with mouse anti-His_5 monoclonal antibody (Qiagen) for the adsorption of His-tagged HRI, or with mouse JJ3 anti-p23 monoclonal antibody (provided from Dr. David Toft), mouse EC1 anti-FKBP52 monoclonal antibody (SRA-1400, Stressgen), or mouse M2 anti- FLAG monoclonal antibody (Sigma). Control reactions lacking template coding for His-tagged HRI (or FLAG-[^{35}S]PP5 for experiments examining PP5 interactions) were used as negative controls for nonspecific binding of chaperone and co-chaperone components from reticulocyte lysate. Unless specified in the figure legends, the immune pellets were washed once with PIPES buffer (10 mM, pH 7.2) containing 150 mM NaCl with 0.5% Tween20, followed by 3 washes with PIPES buffer lacking Tween-20. Samples were separated by SDS-PAGE, and analyzed by electrotransfer to polyvinylidene difluoride membrane (PVDF, Bio-Rad) followed by autoradiography to visualize [^{35}S]-His tagged HRI or FLAG-[^{35}S]PP5. western blotting with polyclonal anti-Hsp90 (41), polyclonal anti-Cdc37 (141), N27 anti-Hsp70 monoclonal (N27F3-4, StressGen), JJ3 anti-p23 monoclonal, F5 anti-HOP monoclonal, and EC1 anti-FKBP52 monoclonal antibodies was used to detect chaperone components co-adsorbed with HRI or with basal Hsp90 complexes adsorbed from lysate using an anti-N-terminus Hsp90 antibody (PA3-013 Affinity BioReagents).

Analysis of the effect of novobiocin on the autokinase activity of HRI. [^{35}S]-His-tagged HRI was synthesized and matured in heme-deficient reticulocyte lysate as described above. After immunoadsorption of His-tagged HRI, its autokinase activity was assayed by incubation of the immune pellet in buffer containing 5 µCi of [γ-^{32}P]-ATP (PerkinElmer) and 2 mM unlabeled ATP for 5 min at 30º C in the presence or absence of novobiocin, as described previously (138, 142). Autophosphorylation of HRI was quantified by scanning densitometry of the autoradiogram screened to eliminate ^{35}S-emmissions. The band intensity of the [^{32}P]-HRI was expressed as optical density (O.D.×mm^2).

Proteolytic fingerprinting of Hsp90. TnT reticulocyte lysate was incubated under conditions for protein synthesis in the absence of plasmid at 30 ºC in the presence of drug or vehicle control for 10 min. For sequential drug treatments, the second drug was applied after the first 5 min of incubation. Reaction mixtures were then chilled on ice and digested with the indicated amount of trypsin at the concentrations indicated in the figure legend as described previously (125). Samples were separated by SDS-PAGE and western blotted using antibodies specific to the N-terminus of Hsp90 (PA3-013 Affinity BioReagents), or the C-terminus of Hsp90 (AC88: kindly provided by Dr. David Toft) (125).

To estimate the Hill coefficient for novobiocin binding, the intensity of the 78 kDa band generated upon incubation of untreated lysate with 24 µg/ml of trypsin and detected by western blotting with the antibody specific for the N-terminus of Hsp90 was assume to represent 0% occupancy of Hsp90's novobiocin binding sites. The change in intensity of the 78 kDa with varying concentrations of novobiocin was used to estimate

the fraction of novobiocin-bound versus free Hsp90 from which the Hill plot was constructed.

Purification and proteolytic fingerprinting of the C-terminal domain of Hsp90 (HSP90-CT). To express Hsp90's C-terminal nucleotide-binding domain, a PCR product encoding amino acids Q531 to D732 of human Hsp90α was amplified using primers that included exogenous sequences to facilitate subcloning, and this PCR product was ligated into pQE32 (Qiagen). The resultant gene product thus included human Hsp90α residues Q531-D732 fused to an N-terminal tag (MRGSHHHHHHGIRM) derived from the vector and linker sequences. This gene product was purified to apparent homogeneity from E. coli lysates using metal-ion affinity chromatography on nickel affinity resin (Qiagen), wherein recombinant Hsp90 gene product was eluted from this resin with imidazole. After elution, peak fractions were pooled and dialyzed against 100 mM ammonium bicarbonate, 1 mM DTT. After dialysis, aliquots were lyophilized and stored in liquid nitrogen until further use.

Purified Hsp90-CT was dissolved with 100 µl (~0.4 µg/ml) of 20 mM Tris HCl pH 7.4. Aliquots of Hsp90-CT (10 µl) were used for each fingerprint assay. Each sample was incubated on ice for 30 min with drug at the concentration indicated in the figure legend. Each sample was digested for 6 min on ice with 25 µl of assay buffer (10 mM Tris HCl pH 7.4, 150 mM NaCl, 4 mM $CaCl_2$, 0.1 mM EDTA) containing trypsin at the concentrations indicated in the figure legend. Reactions were terminated with boiling SDS sample buffer, and analyzed by SDS-PAGE and western blotting with the AC88 antibody which detects an epitope in the C-terminus of Hsp90 (AC88).

Results

Effect of novobiocin on the interaction of Hsc70 and p23 with Hsp90. Hsp90 function is modulated through its interaction with other co-chaperones. Consistent with results reported by Marcu and coworkers (25), novobiocin reduced the amount of Hsc70 and p23 co-immunoadsorbed with Hsp90 from reticulocyte lysate in a concentration dependent manner (Fig. 4A). While the concentration required to reduce the interaction of Hsc70 and p23 with Hsp90 was somewhat higher than those reported by Marcu *et al* (25), these results confirm the ability of novobiocin to alter Hsp90's default interactions with its co-chaperone partners.

Effect of novobiocin on the maturation and activation of HRI. The maturation and activation of HRI in heme-deficient reticulocyte lysate (HRI "transformation") requires its interaction with Hsp90 and its co-chaperone Cdc37 (41, 138). Under normal conditions in reticulocyte lysate, Hsp90 and Cdc37 form a high-affinity complex with protein kinases after their release from the ribosome that is stable in the presence of high salt (41, 141). Geldanamycin inhibits Hsp90-facilitated transformation of newly synthesized HRI in reticulocyte lysate (41, 138), prevents the formation of salt-stabile high-affinity complexes between Hsp90 and HRI, and blocks the incorporation of Cdc37 into complexes formed between Hsp90 and newly synthesized HRI (41, 141). On the other hand, molybdate in the presence of ATP, "freezes" Hsp90-client complexes, renders Hsp90-client complexes stable to washing with high salt, but also blocks the transformation of HRI (41, 141). Therefore, we investigated whether novobiocin had the capacity to inhibit the ability of Hsp90 to facilitate the transformation of newly

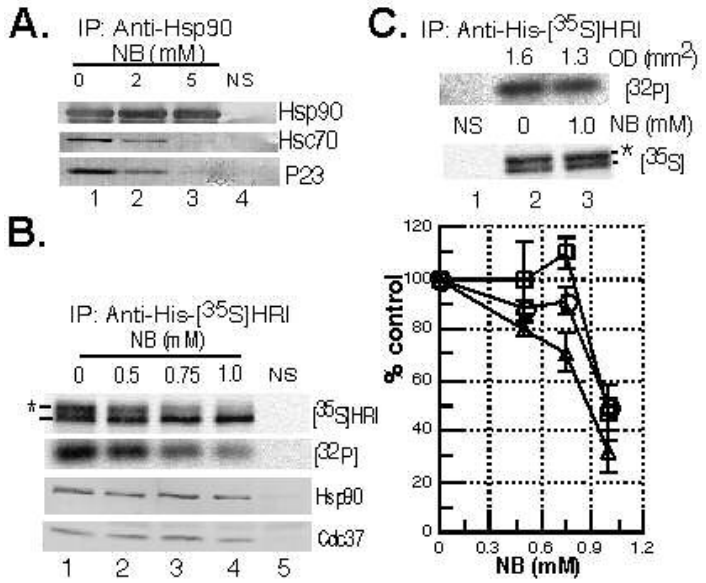


Figure 4. Effect of novobiocin on protein folding and Hsp90/co-chaperone interactions in rabbit reticuocyte lysate. (**A**) Nuclease-treated reticulocyte lysate was incubated on ice in the absence of an ATP-regenerating system with 0, 2 or 5 mM novobiocin for 1 h. Samples were immunoadsorbed with anti-Hsp90 antibody and analyzed by SDS-PAGE and western blotting for Hsp90, and co-adsorbed Hsc70 and p23. (**B**) [^{35}S]-His-tagged HRI was synthesized in TnT reticulocyte lysate and then matured in normal heme-deficient lysate in the presence or absence (buffer control) of 0.5, 0.75, 1.0 mM novobiocin as described under "Experimental Procedures". His-tagged HRI was immunoadsorbed and samples were analyzed by SDS-PAGE, and autoradiography ([^{35}S]HRI and [^{32}P]HRI, upper two panels) and western blotting (Hsp90 and Cdc37). His-tagged HRI was assayed for autokinase activity as described under "Experimental Procedures". NS: Analysis of sample lacking template coding for His-tagged HRI as a control for nonspecific binding of Hsp90 and Cdc37 to the immune resin (B, left hand panels). Band densities of [^{32}P]HRI (open triangles) from the autoradiogram, and Hsp90 (open circles) and Cdc37 (open squares) from the western blots were quantified by densitometry (O.D.×mm^2) and plotted as percent of the buffer control versus novobiocin concentration (average of three experiments ± standard deviation) (B, right hand panel). (**C**) His-tagged HRI was synthesized and matured in heme-deficient reticulocyte lysate as described above. Immunoadsorbed HRI was assayed for autokinase activity in the presence (1 mM, lane 3) or absence (0 mM, lane 2) of novobiocin as described under "Experimental Procedures". Band intensity of [^{32}P]-HRI was quantified by densitometry, and expressed as OD×mm^2. NS: Analysis of sample lacking template coding for His-tagged HRI as a control for nonspecific binding of any endogenous HRI. Autoradiogram of [^{35}S]-HRI (lower panel) and autophosphorylated [^{32}P]-HRI (upper panel).

synthesized HRI, and whether it similarly altered the interactions of Hsp90 and Cdc37 with HRI.

[^{35}S]-His-tagged HRI was synthesized in reticulocyte lysate and subsequently matured in heme-deficient lysate in the presence or absence of novobiocin. The Hsp90/Cdc37-dependent transformation of HRI resulted in the autophosphorylation of HRI and the appearance of a species of HRI with slower electrophoretic mobility during SDS-PAGE [Fig. 4B, and (41, 138)]. Novobiocin inhibited the "transformation" of HRI in a dose dependent fashion, as evidence by the decrease in amount of [^{35}S]-HRI that exhibited slower electrophoretic mobility (Fig. 4B).

The effect of novobiocin on the co-immunoadsorption of Hsp90 and Cdc37 with HRI was examined to determine whether novobiocin disrupted the interactions of Hsp90 and Cdc37 with HRI. Western blotting indicated that there was a dose dependent decrease in the amounts of Hsp90 and Cdc37 that were co-adsorbed with His-tagged HRI from heme-deficient lysates (Fig. 4B). Consistent with our previous work (138), reciprocal co-immunoadsorptions of HRI with anti-Hsp90 or anti-Cdc37 antibodies from novobiocin-treated lysate indicated that only the faster migrating species of HRI (e.g., untransformed HRI) was associated with Hsp90 and Cdc37 (not shown). Hsp90/Cdc37-dependent transformation of HRI in heme-deficient lysate is accompanied by the activation of HRI's autokinase activity. Therefore, we measured the effect of novobiocin on the autophosphosphorylation of HRI (Fig. 4B, left panel). Again novobiocin inhibited the activation of HRI's autokinase activity in a concentration dependent manner. Quantification of the degree of novobiocin-induced inhibition of HRI's autophosphorylation indicated that it correlated closely with the extent of

novobiocin-induced reduction of the interaction of Hsp90 and Cdc37 with HRI (Fig. 4B): the concentrations of novobiocin that were required to inhibit the interaction of Hsp90 and Cdc37 with HRI and to inhibit HRI's autokinase activity by 50% were both between 0.75 and 1 mM (Fig. 4B).

Effect of novobiocin on the autophosphorylation of HRI. Autophosphorylation of HRI is required for its transformation into an active Hsp90-independent kinase (41, 138). Because novobiocin inhibits DNA gyrase through its ability to bind at or near DNA gyrases's ATP binding pocket, we examined the effect of novobiocin on the autophosphorylation of transformed HRI *in vitro* at quasi-physiological ATP concentrations (e.g., 2 mM). Addition of 1 mM novobiocin to HRI autokinase assays had little direct effect on HRI's autokinase activity *in vitro* (Fig. 4C), indicating that novobiocin did not compete with ATP for binding to HRI. Thus, there appears to be a good correlation between the ability of novobiocin to reduce the interaction of Hsp90 and Cdc37 with newly synthesized HRI, and its ability to inhibit the Hsp90/Cdc37-dependent activation of HRI's autokinase activity.

Effect of novobiocin on the stability of the interaction of Hsp90/Cdc37 with HRI. The strength of the interactions between Hsp90, Cdc37 and newly synthesized HRI is regulated through nucleotide-modulated conformational switching of Hsp90 (41, 141). To further characterize the effect of novobiocin on the interactions of Hsp90 and Cdc37 with HRI, we examined the effect of novobiocin on the salt-stability of complexes formed between Hsp90, Cdc37, and HRI. Newly synthesized His-tagged HRI was immunoadsorbed from control and drug-treated reticulocyte lysate, and immunoresins were washed with buffer containing a low or high concentration of NaCl (Fig. 5).

Western blot analysis indicated that in the absence of drug, high affinity complexes were formed between Hsp90/Cdc37 and newly synthesized HRI, which were stable in the presence of high salt concentrations (Fig. 5A, 0). Consistent with previous results (41, 141), the presence of molybdate similarly led to the formation of salt-stable Hsp90/Cdc37-HRI complexes (Fig. 5A, MoO_4), while geldanamycin blocked the interaction of Cdc37 with Hsp90-kinase complexes, and rendered the interaction of Hsp90 with HRI salt-labile (Fig. 5A, GA). In contrast, both Hsp90 and Cdc37 were co-adsorbed with HRI from reticulocyte lysate treated with 1 mM novobiocin, and their association with HRI was not affected upon washing with buffer containing high salt. However, neither Hsp90 nor Cdc37 were co-adsorbed with HRIfrom lysate treated with 2 mM novobiocin, indicating that novobiocin can completely disrupt the interaction of Hsp90 with a client target at concentrations $\geq$ 2 mM. These results suggest that novobiocin has effects on Hsp90/Cdc37/kinase interactions that are distinct from those induced by molybdate and geldanamycin.

Effect of novobiocin on molybdate-induced stabilization of Hsp90-Cdc37-kinase complexes. As noted above, newly synthesized protein kinases form high-affinity complexes with Hsp90 and Cdc37 that are stabile to washing in buffers containing high salt (e.g., 0.5 M NaCl) immediately after their release from the ribosome (129, 141). Furthermore, addition of molybdate in the presence of ATP "freezes" these high affinity complexes preventing kinase maturation and activation, while the addition of geldanamycin prevents the formation of these high-affinity Hsp90-Cdc37-kinase complexes (129, 141). Order-of-addition experiments have indicated that pre-incubation

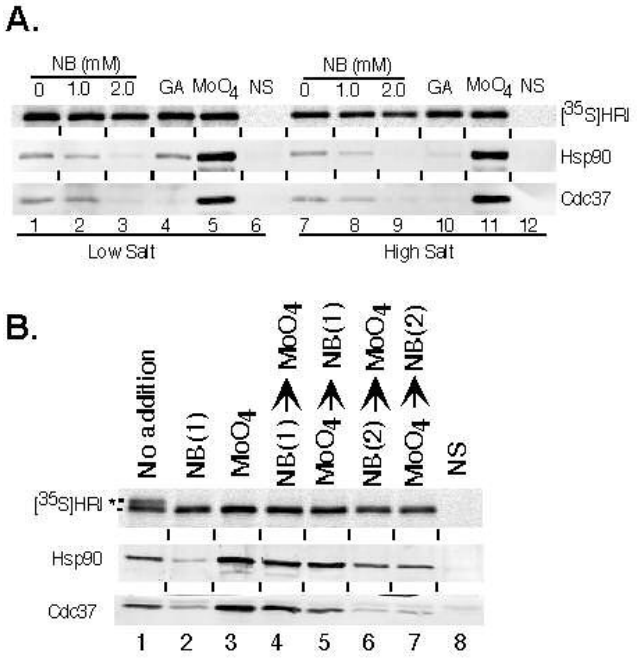


Figure 5. Effects of pharmacological agents on the salt stability of the interaction of Hsp90-Cdc37 with HRI. (**A**) [^{35}S]-His-tagged HRI was synthesized in TnT reticulocyte lysate as described under "Experimental Procedures". Ten minutes after arresting initiation of translation, HRI was matured in the absence (0 mM, lanes 1 and 7) or presence of 1 mM (lanes 2 and 8) or 2.0 mM (lanes 3 and 9) novobiocin, 10 µg/ml:18 µM geldanamycin (GA, lanes 4 and 10), or 20 mM sodium molybdate (MoO_4, lanes 5 and 11) for 1 h. His-tagged HRI was immunoadsorbed, followed by washing of the immunoresins with 10 mM PIPES buffer (pH 7.2) containing no salt (lanes 1 – 6) or 500 mM NaCl (lanes 7 – 12). (**B**) [^{35}S]-His-tagged HRI was synthesized in TnT reticulocyte lysate and subsequently matured in normal heme-deficient lysate that had been pre-treated with buffer (lane 1), 1.0 mM novobiocin (NB(1), lanes 2 and 4), 2.0 mM novobiocin (NB(2), lane 6), or 20 mM sodium molybdate (MoO_4, lanes 3, 5 and 7) for 5 min. After 5 min of incubation at 30 °C, samples were supplemented with buffer (lanes 1-3), 1.0 mM novobiocin [NB(1), lane 5], 2 mM novobiocin [NB(2), lane 7] or 20 mM sodium molybdate (MoO_4, lanes 4 and 6). After incubation for 60 min, His-tagged HRI was immunoadsorbed, and samples were washed as described under "Experimental Procedures". Samples were separated by SDS-PAGE, and analyzed by autoradiography ([^{35}S]HRI) and western blotting (Hsp90, middle panel; Cdc37, lower panel). NS: Analysis of sample lacking template coding for His-tagged HRI as a control for nonspecific binding of Hsp90 and Cdc37 to the immune resin (A: lanes 6 and 12; B: lane 8).

of reticulocyte lysate with either geldanamycin or molybdate blocks the pharmacological effect of the other upon its subsequent addition to lysate (141). The ability of novobiocin to induce dissociation of Hsp90-Cdc37 from immature HRI molecules suggests that the binding of novobiocin to Hsp90 induces Hsp90 to adopt a conformation distinct from the conformation Hsp90 adopts in the presence of ATP and molybdate. Thus, we carried out order-of-addition experiments to determine whether molybdate or novobiocin had a dominant effect to the other upon its pre-incubation in reticulocyte lysate (Fig. 5B). While the presence of molybdate stabilized the interaction of Hsp90 and Cdc37 with HRI, a reduction in the binding of Hsp90 and Cdc37 to HRI was apparent in the presence of 1 mM novobiocin and molybdate, and a marked reduction in the interaction between these proteins occurred in the presence of 2 mM novobiocin. Furthermore, this reduction in binding was observed whether molybdate was present in the initial pre-incubation (Fig. 5B, lane 7) or whether it was added to lysate after pre-incubation with novobiocin (Fig. 5B, lane 6). Thus, binding of novobiocin and molybdate to Hsp90 does not appear to be mutually exclusive and may occur concurrently. In addition, it was quite clear that novobiocin inhibited the ability of molybdate to stabilize the binding of Hsp90 and Cdc37 to its client target, HRI.

Effect of novobiocin on the basal interaction of co-chaperones with Hsp90. Marcu and co-workers have reported that Hsp70 and p23 are not co-adsorbed with Hsp90 from novobiocin-treated reticulocyte lysate (26). However, their experiment was carried out on ice and it was not apparent from the description of the experimental protocol whether an ATP-regenerating system was present. Therefore, we further characterized the effect of novobiocin on basal complexes formed between Hsp90 and its

co-chaperones in reticulocyte lysate. Anti-Hsp90 immunoadsorptions were carried out on ice, or at 30 ºC in the presence of an ATP regenerating system and analyzed for co-adsorbing Hsc70, HOP, FKBP52, Cdc37 and p23. On ice, 4 and 10 mM novobiocin significantly reduced the interaction of Hsc70, FKBP52 and p23 with Hsp90, while it suppressed the interaction of HOP with Hsp90 to a small degree and had little effect on the amount of Cdc37 that was co-adsorbed with Hsp90 (Fig. 6A).

In the presence of an ATP-regenerating system, Hsp90 is a mixture of molecules in their default conformation, and those that are actively cycling after binding to cryptic client substrates. In the absence of molybdate, 5 mM novobiocin markedly reduced the binding of Hsc70 to Hsp90, with 10 mM novobiocin blocking their interaction completely (Fig. 6B). Both concentrations of novobiocin inhibited the interaction of p23 and Hsp90. The interaction Cdc37 with Hsp90 was affected very little in the presence of 5 mM novobiocin, while 10 mM novobiocin reproducibly caused a slight increase in the binding of Cdc37 to Hsp90. On the other hand, the interaction of HOP with Hsp90 was decreased by 13 ± 9% and 44 ± 19% (mean ± s.d. of three experiments) in the presence of 5 and 10 mM novobiocin, respectively, but was unaffected by 2 mM novobiocin (not shown).

Addition of molybdate to reticulocyte lysate in the presence of the ATP regenerating system caused a significant decrease in the amount of Hsc70 that bound

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interaction of HOP with Hsp90 (Fig. 6B, lane 4 versus 1). In contrast, molybdate

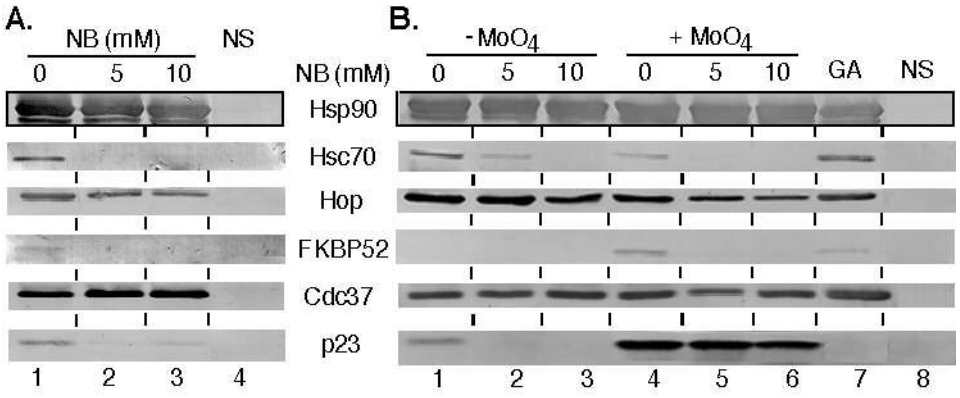

Figure 6. Effects of novobiocin on the basal interaction of co-chaperones with Hsp90. (A) Nuclease-treated TnT reticulocyte lysate was incubated without the addition of an ATP-regenerating system in the absence (lane 1) or the presence of 5 mM (lane 2) or 10 mM (lane 3) novobiocin on ice for 1 h. (B) Nuclease-treated TnT reticulocyte lysate was incubated under conditions for coupled transcription-translation for 10 min at 30 °C with no additions (lane 1), 5 mM (lane 2) or 10 mM (lane 3) novobiocin, 20 mM molybdate (lane 4), or 20 mM molybdate and 5 mM (lane 5) or 10 mM (lane 6) novobiocin, or 10 µg/ml geldanamycin (GA, lane 7). Hsp90 was immunoadsorbed (IP: boxed panel) and samples were separated by SDS-PAGE, and analyzed by western blotting for Hsp90, Hsc70, HOP, FKBP52, Cdc37 and p23. NS: Sample prepared using a non-specific IgG as a control for nonspecific binding of proteins to the immune resin (A: lane 4; B: lane 8). Boxed panels indicates the target protein for the immunoadsorption.

increased in the amounts of FKBP52 and p23 bound to Hsp90 (Fig. 6B). This is consistent with molybdate's ability to stabilize late Hsp90 heterocomplexes that lack Hsc70 and HOP, but contain p23 and FKBP52. In the presence of molybdate, novobiocin eliminated the binding of both Hsc70 and FKBP52 to Hsp90. The 5 mM and 10 mM concentrations of novobiocin further decreased the interaction of HOP with Hsp90 by approximately 23 ± 14% and 55 ± 9%, respectively (mean ± s.d. relative to the molybdate control of three experiments). In contrast, novobiocin at concentrations of 5 mM and 10 mM had only a slight inhibitory effect on the interaction of Cdc37 with Hsp90 (Fig. 6B). Furthermore, in the presence of molybdate, novobiocin had little effect on the interaction of p23 with Hsp90, with 10 mM novobiocin causing a slight but reproducible reduction in the interaction of p23 with Hsp90. Again the effects of novobiocin on the basal interactions of Hsp90 with its co-chaperones were distinct from those of geldanamycin: geldanamycin blocked the association of p23 with Hsp90, reduced the amount of HOP that was co-adsorbed with Hsp90, but had little effect on the interactions of Hsc70, FKBP52 and Cdc37 with Hsp90 (Fig. 6B, GA).

Effect of novobiocin on the interaction of p23, FKBP52 and PP5 with newly synthesized HRI. FKBP52, protein phosphatase 5 (PP5) and p23 are co-chaperones that interact with Hsp90-client complexes that are formed "late" in the ATP-driven reaction cycle of Hsp90 [reviewed in (13, 82, 101)]. Recently, we have demonstrated that co-chaperone components containing tetratricopeptide repeat (TPR) motifs (i.e. FKBP52 and PP5), which interact with the C-terminal EEVD motif of Hsp90 (49, 60, 61, 69, 70, 143, 144), can be present as components of Hsp90-Cdc37 complexes containing bound kinase (129, 141). Thus, to characterize further the step in the reaction cycle that is affected by

novobiocin, we examined the effect of novobiocin on the interactions of the co-chaperones p23, PP5 and FKBP52 with client, HRI, and the results were compared with the effects of molybdate and geldanamycin on these interactions.

Newly synthesized His-tagged-HRI was immunoadsorbed from heme-replete reticulocyte in the presence and absence of drug treatments (Fig. 7A), and the co-adsorption of Hsp90, Cdc37. FKBP52, and p23 with HRI were assessed by western blotting. Consistent with previous results (129, 141), geldanamycin completely disrupted the interaction of Cdc37 with HRI, while a weak interaction of Hsp90 with HRI was maintained (Fig. 7A: GA). Geldanamycin also inhibited the co-adsorption of p23 and FKBP52 with His-tagged HRI. In contrast, 2 mM novobiocin completely disrupted the binding of both Hsp90 and Cdc37 to HRI (Fig. 7A). Interestingly, western blotting indicated that 2 mM novobiocin reproducibly increased the amount of FKBP52 that was co-adsorbed with His-tagged HRI (Fig. 7A). In addition, p23 (Fig. 7A, lane 1) was detected to co-adsorb with His-tagged HRI. In the presence of 2 mM novobiocin, however, the amount of p23 that was co-adsorbed with HRI was reduced to a nearly imperceptible level (not readily visible on scanned data: Fig 4A, lane 2). Co-adsorption of p23 with HRI was readily detectable in the presence of molybdate, and the level of co-adsorbed p23 was decreased markedly in the presence of 2 mM novobiocin (Fig. 7A, lane 3 *versus* 4). Currently, antibodies available to PP5 do not have sufficient sensitivity to detect PP5.Immunoadsorptions of His-tagged HRI (Fig. 7B, C and D) from heme-deficient lysate were also carried out. Novobiocin and geldanamycin both inhibited the transformation of HRI in heme-deficient lysate as evidenced by the lack of an [^{35}S]-HRI exhibiting slower electrophoretic mobility on SDS PAGE (Fig. 7B, C and D: left panels).

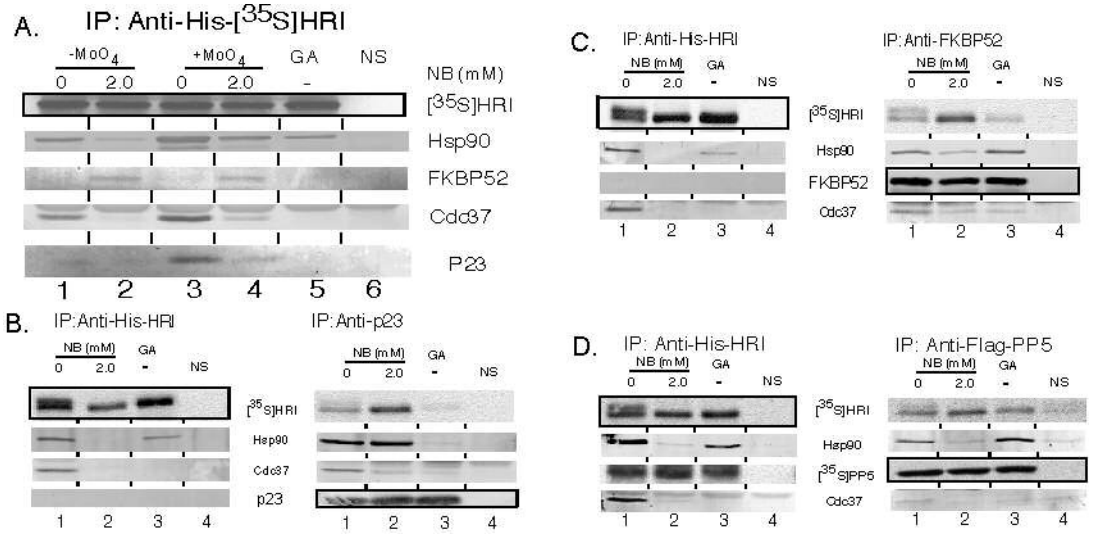

Figure 7. Effects of novobiocin on the interaction of HRI with Hsp90 co-chaperones. (**A**) [^{35}S]-His-tagged HRI was synthesized in TnT reticulocyte lysate. After inhibition of initiation by the addition of aurintricarboxylic acid, the lysate was incubated for 1 h at 30 °C with no additions (lane 1), 2 mM novobiocin (lane 2), 20 mM molybdate (lane 3), 2 mM novobicin plus 20 mM molybdate (lane 4), or 10 µg/ml geldanamycin (GA, lane 5). (**B**) [^{35}S]-His-tagged HRI was synthesized in TnT reticulocyte lysate and then matured in normal heme-deficient lysate in the absence (B-D: lane 1) or presence of 2 mM novobiocin (B-D: lane 2) or 10 µg/ml geldanamycin (B-D: GA, lane 3), as described under "Experimental Procedures". For the experiment shown in part D of the figure, sample containing His-tagged HRI was mixed with an equal volume of lysate containing newly synthesized FLAG-[^{35}S]PP5 prior to dilution of the sample into heme-deficient lysate. Samples for (A) were immunoadasorbed with anti-His-tag antibody, while samples for B-D) were divided in half and immunoadsorbed with anti-His-tag (B-D, left panel), JJ3 anti-p23 (B: right panel), EC1 anti-FKBP52 (C: right panel) or M2 anti-FLAG (D: right panel) antibody. After washing, the samples were separated by SDS-PAGE, and analyzed by autoradiography (A-D: [^{35}S]HRI, upper panel; D, [^{35}S]PP5) and western blotting (Hsp90, FKBP52, Cdc37 and p23). Boxed panels indicates the target protein for the immunoadsorption. NS: Analysis of sample lacking template coding for His-tagged HRI as a control for nonspecific binding of Hsp90 and Cdc37 to the immune resin (A: lane 6; B-D lane4, all panels).

The effects of novobiocin and geldanamycin on the co-adsorption of Hsp90 and Cdc37 (Fig. 7B, C, And D: left panels) were the same as those observed for the experiments carried out in heme-replete lysate (Fig. 7A). However, the amount of HRI immunoadsorbed in these experiments was not sufficient to detect co-adsorption of either p23 or FKBP52.

Immunoadsorptions of p23 (Fig. 7B: right panel), FKBP52 (Fig. 7C: right panel) and [^{35}S]-FLAG-PP5 (Fig. 7D: right panel) from heme-deficient reaction mixes were carried out in parallel to overcome the poor detection sensitivity of antibodies directed against these proteins on western blots. Geldanamycin markedly reduced the amount of Cdc37 that was co-adsorbed with basal complexes containing p23 (Fig. 7B: right panel), FKBP52 (Fig. 7C: right panel) or PP5 (Fig. 7D: right panel). While geldanamycin blocked the basal interaction of p23 with Hsp90 (Fig. 7B: right panel), it had little effect on the basal interaction of FKBP52 (Fig. 7C: right panel) and PP5 (Fig. 7D: right panel) with Hsp90. Furthermore, while little HRI was co-adsorbed with p23 from geldanamycin-treated lysate, geldanamycin only partially reduced the amount of HRI that was co-adsorbed with FKBP52 and Flag-PP5. The co-adsorption of HRI with Flag-PP5 in the presence of geldanamycin is consistent with our previous work (45, 129).

In contrast to the effects of geldanamycin, HRI was co-immunoadsorbed in conjunction with p23, FKBP52 and Flag-[^{35}S]-PP5 from lysates treated with 2 mM novobiocin. Western blotting of samples from anti-co-chaperone immunoadsorptions (Fig. 7B, C and D: left panels) indicated that 2 mM novobiocin markedly reduced the amount of Cdc37 present in basal complexes with p23, and nearly completely blocked the interaction of Cdc37 with basal complexes containing FKBP52 or PP5. In addition,

2 mM novobiocin brought about a reduction in the amount of Hsp90 present in basal complexes with FKBP52 and PP5, but had only a marginal effect on the basal interaction of p23 with Hsp90. Data presented in Figure 4 suggests that novobiocin concentrations of greater than 2 mM may be required to quantitatively disrupt the basal interaction of p23 with Hsp90. Thus, the data suggests that the p23, FKBP52 and PP5 co-chaperone components of the Hsp90 chaperone machine can maintain their interaction with HRI after novobiocin-induced dissociation of Hsp90 and Cdc37 from this client target. Since these co-chaperones are components of "late" complexes formed between the Hsp90 chaperone machine and client targets, the data suggest that novobiocin may bind Hsp90 during or subsequent to the formation of these late complexes.

Effect of novobiocin on the conformation of Hsp90. Hsp90 gives different proteolytic fingerprints in its geldanamycin-bound and its molybdate-bound states (141). The proteolytic fingerprint of Hsp90 in the presence of geldanamycin is the same as the fingerprint of Hsp90 in its default conformation. Order-of-addition experiments have indicated that the geldanamycin-bound and molybdate-bound conformations are not freely interchangeable, such that Hsp90 adopts the conformation induced by the first agent added, and does not convert to the alternate conformation upon the subsequent addition of either geldanamycin or molybdate (141). The biochemical properties of geldanamycin-bound Hsp90 suggest that Hsp90 is in its "open" conformation and poised to bind client, while the properties of molybdate-bound Hsp90 formed in the presence of ATP, suggest that Hsp90 is in its "closed" conformation: a conformation equivalent to the salt-stabile high affinity conformation Hsp90 adopts with bound client [e.g., with newly synthesized HRI (141)] in the presence of hydrolysable ATP(77, 118, 125, 131) .

Proteolytic fingerprinting of Hsp90 *in situ* in reticulocyte lysate was carried out to determine whether the binding of novobiocin induces Hsp90 to adopt a conformation that differs from the conformation it assumes upon binding of geldanamycin or molybdate (Fig. 8). Membranes were blotted with antibodies directed against N-terminal residues of Hsp90 or C-terminal residues of Hsp90 (AC88) (145) to determine the position of the major cut sites (141). The trypsinolytic fingerprint of Hsp90 generated in the presence of 2 mM novobiocin was indistinguishable from the fingerprints of Hsp90 generated from untreated lysate (its default conformation, Fig. 8A). Addition of geldanamycin either before or after incubation of lysate with 2 mM novobicin also had no effect on Hsp90's fingerprint (data not shown). However, novobiocin concentrations of greater than 5 mM had significant effects on Hsp90's proteolytic fingerprint. At high novobiocin concentrations, a 50 kDa C-terminal fragment was detected by western blotting with the AC88 antibody (Fig. 8A right panel). This fragment is also generated when molybdate alone is added to reticulocyte lysate (Fig. 8B). In addition, the 30, kDa, 50 kDa doublet and the 78 kDa N-terminal fragments of Hsp90 were lost with increasing novobiocin concentrations and a new 73 kDa fragment appeared (Fig. 8A left panel). Consistent with our previously published results (141), addition of molybdate altered the fingerprint of Hsp90, with the binding of molybdate protecting Hsp90 from cleavage by trypsin at a site around amino acid 400 and decreasing the rate of cleavage at a site around amino acid 600, such that a major stable 50 kDa fragment representing the C-terminal half of Hsp90 was detected on western blots with the AC88 anti-Hsp90 antibody (Fig. 8B: right panel). Addition of 0.5 to 5 mM novobiocin in the presence of molybdate lead to a concentration dependent protection of Hsp90 from trypsinolytic cleavage at the site near amino acid

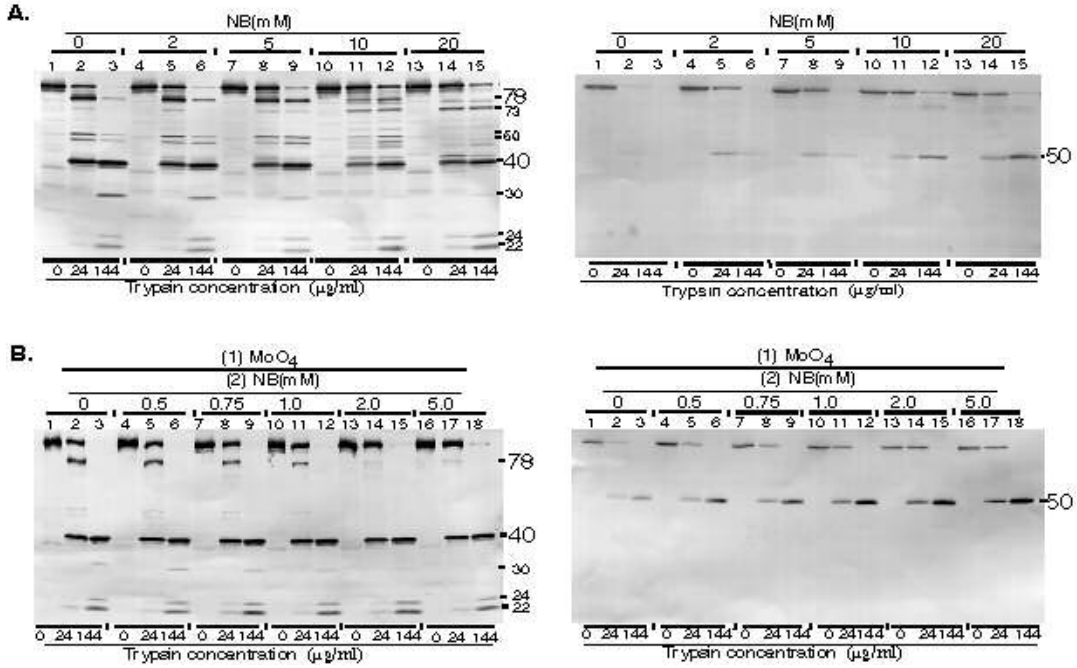

Figure 8. Proteolytic mapping of structural changes in Hsp90 induced by novobiocin. TnT reticulocyte lysate was incubated under conditions for protein synthesis in the absence of plasmid for 10 min at 30 ºC in the presence of the indicated concentrations of novobiocin (**A**: 0, 2, 5, 10 and 20 mM) or in the presence of 20 mM molybdate plus the indicated concentrations of novobiocin (**B**: 0, 0.5, 0.75, 1, 2 and 5 mM). Reactions were chilled on ice and incubated for 6 min in the presence of the indicated concentration of trypsin and analyzed as described under "Experimental Procedures". Western blot with AC88 anti-Hsp90 antibody which recognizes an epitope within the C-terminal region of Hsp90 [AC88(α-CT): A and B- right panel]. Western blot with anti-Hsp90 antibody raised to sequence at the N-terminus of Hsp90 [ABR(α-NT): A and B- left panel].

600, leading to the disappearance of the 78 kDa Hsp90 fragment detected by the anti-N-terminal Hsp90 antibody (Fig. 8B: left panel) and an increase in the amount of 50 kDa C-terminal fragment detected by the AC88 anti-Hsp90 antibody (Fig. 8B: right panel). Furthermore, a 30 kDa N-terminal fragment disappeared from Hsp90 fingerprints generated in the presence of molybdate and increasing concentrations of novobiocin (Fig. 8B: left panel). Consistent with data presented in Figure 5, the order of addition of molybdate and novobiocin had no effect on the trypsinolytic fingerprints of Hsp90 (data not shown). Thus, in the presence of molybdate and novobiocin, only the major cleavage site near amino acid 280 and two minor sites around amino acid 230 of Hsp90 were cleaved, generating 40 kDa and 22/24 kDa bands detected by blotting with antibody specific to the N-terminus of Hsp90 (Fig. 8B: left panel). Cleavage near amino acids around 230 and 280 represent cutting of Hsp90 near the beginning and the end of the charged linker region that separates the N-terminal ATP binding domain of Hsp90 from its middle domain (146).

Results presented in Figure 4 indicated that novobiocin-induced inhibition of HRI's Hsp90/Cdc37 dependent acquisition of autokinase activity correlated directly with disruption of the binding of Hsp90 and Cdc37 to HRI. Therefore, the decrease in the intensity of the 78 kDa N-terminal fragment and the increase in the intensity of the 50 kDa C-terminal fragment was quantified and used to estimate the change in Hsp90 conformation induced by increasing concentrations of novobiocin (Fig. 9A). The concentration of novobiocin required to induce a 50% changed in the cleavage of Hsp90 was approximately10 mM. A Hill plot, constructed assuming that the change in intensity of the 78 kDa band induced by novobiocin was proportional to the amount of novobiocin-

bound Hsp90, yielded a Hill coefficient of 1.7 (R = 0.98) suggesting that the binding of novobiocin is highly cooperative.

However, in the presence of molybdate, only ~1 mM novobiocin was required to induce a 50% change in the cleavage of Hsp90 (Fig. 9A). Thus, the conformational change in Hsp90 that is induced by the binding of molybdate in the presence of ATP increased the binding affinity of Hsp90 for novobiocin by ten fold. The change in Hsp90 conformation induced by novobiocin in the presence of molybdate correlated well with novobiocin-induced disruption of the interaction of Hsp90 and Cdc37 with HRI and inhibition of HRI's autokinase activity (Fig. 4B). These results suggest that there may be a relationship between the conformational change induced in Hsp90 upon the binding of novobiocin to molybdate bound Hsp90 and the dissociation of Hsp90-Cdc37 from complexes containing client kinase. It should be noted that in the presence of molybdate and 5 mM novobiocin, approximately 20% of the 78 kDa band remained relative to the molydate control, suggesting that there may be a population of Hsp90 molecules present in reticulocyte lysate that may not be capable of interacting with molybdate.

To further investigate the effect of novobiocin on the conformation of Hsp90, we examined the ability of the AC88 anti-Hsp90 antibody to immunadsorb Hsp90 from reticulocyte lysate. AC88 does not appear to adsorb Hsp90 present in complexes containing bound client (147), and molybdate decreases the ability of the AC88 antibody to immunoadsorb Hsp90 from reticulocyte lysate (125). The AC88 anti-Hsp90 antibody quantitatively adsorbed Hsp90 from control, geldanamycin-treated, and novobiocin-treated (2 mM) lysate (Fig. 9B). In the presence of molybdate, approximately 50% of the Hsp90 present in reticulocyte lysate was adsorbed by the AC88 antibody, while in the

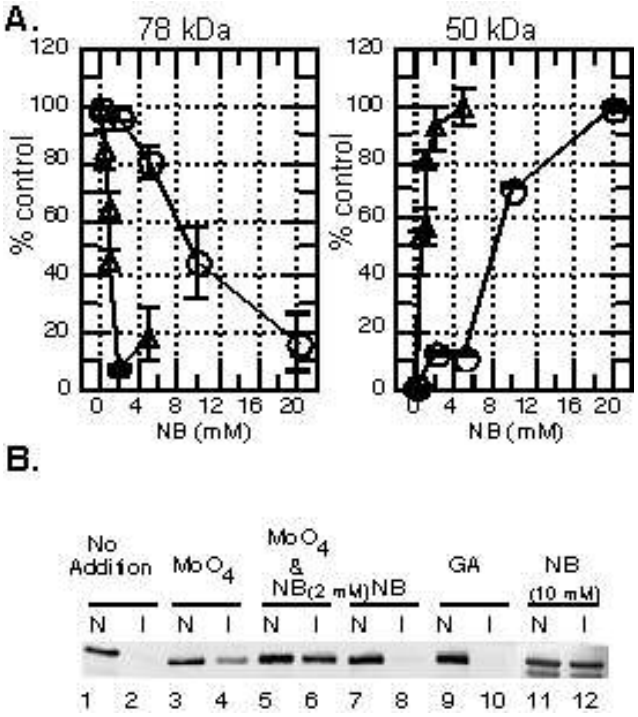

Figure 9. **Structural changes in Hsp90 induced by novobiocin.** (**A**) The amount of the 50 kDa band in the right panels, and the 78 kDa band in left panels from Figure 8A (open circles) and Figure 8B (open triangles) were quantified by scanning densitometry, and plotted as percent of the no novobiocin control (from Figure 8, A and B: 78 kDa band; left panel, lane 2 = 100%), or percent of the maximum observed change (from Figure 8, A and B: 50 kDa band, right panel, lane 15 = 100%). Values are the average of two experiments with the error bars indicating the range of the two points. (**B**) Ability of the AC88 anti-Hsp90 antibody (I) or non-immune control antibody (N) to immunadsorb Hsp90 from rabbit reticulocyte lysate was determined. Briefly, TnT reticulocyte lysate was incubated under conditions for protein synthesis in the absence of plasmid for 20 min at 30 °C, followed by a 10 min incubation in the presence of no additions (lanes 1 and 2), 20 mM sodium molybdate (lanes 3 and 4), 20 mM sodium molybdate and 2 mM novobiocin (lanes 5 and 6), 2 mM novobiocin (lanes 7 and 8), 10 µg/ml geldanamycin (lanes 9 and 10), or 10 mM novobiocin (lanes 11 and 12). After immunoadsorption of Hsp90, the amount of unadsorbed Hsp90 remaining in the unbound fraction was analyzed by SDS-PAGE and western blotting.

presence of both molybdate and novobiocin or high concentrations of novobiocin alone (10 mM) only about 5 to 15% of the Hsp90 was immunoadsorbed. Thus, overall these results suggest that novobiocin interacts with Hsp90 complexes after Hsp90 adopts its molybdate-bound conformation: a conformation with properties similar to those exhibited by Hsp90 after it has formed salt-stabile high-affinity complexes with kinase client.

Novoboicin directly protects the C-terminal domain of Hsp90 from cleavage by trypsin. The effect of novobiocin on the sensitivity of Hsp90 to proteolytic cleavage could result from a direct effect of the binding of novobiocin to Hsp90, or it could be due to novobiocin altering the interaction of co-chaperones with Hsp90. To distinguish between these two possibilities, we examined the effect of novobiocin on the proteolysis of Hsp90's C-terminal domain (amino acids 584-730). The C-terminal domain of recombinant Hsp90 binds novobiocin in the absence of the N-terminal domain of Hsp90 (26). Thus, use of His-tagged recombinant Hsp90-CT domain avoids potential problems caused by the allosteric regulation of the novobiocin binding site via the binding of nucleotide to the N-terminal domain of Hsp90 (27). The recombinant Hsp90-CT was highly sensitive to digestion with trypsin in the absence of novobiocin (Fig. 10A). As expected, geldanamycin, which binds to the N-terminal, but not to the C-terminal domain of Hsp90, had no effect on the protease sensitivity of the domain. Furthermore, molybdate did not protect the Hsp90-CT from cleavage by trypsin. However, novobiocin alone protected recombinant Hsp90-CT from trypsin cleavage, and molybdate had no additional effect on this protection. The ability of MgATP to similarly protect recombinant Hsp90-CT from trypsinolytic cleavage (Fig. 10B) supports the hypothesis that novobiocin inhibits Hsp90 function by binding to a nucleotide binding site present in

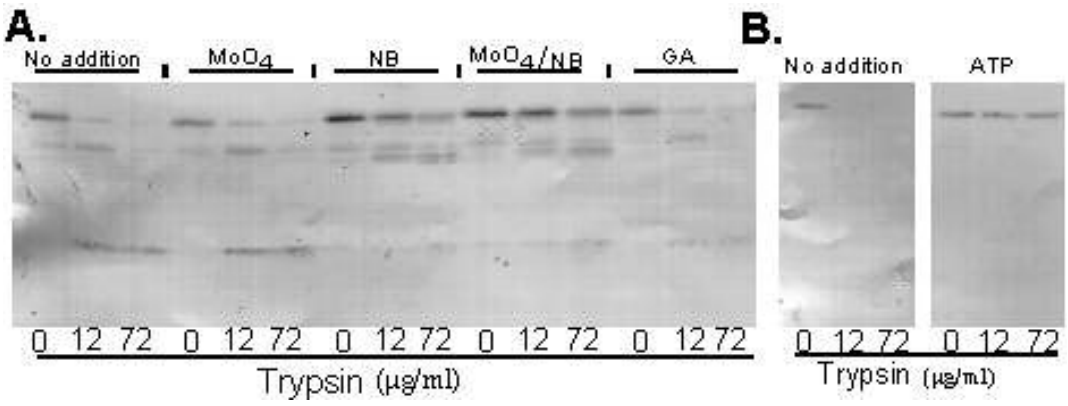

Figure 10. Proteolytic mapping of C-terminal domain of Hsp90. Purified recombinant C-terminal domain was incubated for 10 min at 30 ºC in the presence of (**A**) no additions, 20 mM sodium molybdate (MoO_4), 2 mM novobiocin (NB), 20 mM sodium molybdate and 2 mM novobiocin (MoO_4/NB) or 20 µg/ml geldanamycin (GA), or (**B**) no additions or 5 mM MgATP. Reactions were chilled on ice and incubated 6 min in the presence of the indicated concentration of trypsin and analyzed by SDS-PAGE and western blotting with AC88 anti-Hsp90 antibody.
the C-terminal domain of Hsp90 and altering the structure near its binding site.

Discussion

In this manuscript, we demonstrate that novobiocin inhibits Hsp90-dependent maturation of newly synthesized client target (e.g., the maturation and activation of the Hsp90-Cdc37-dependent kinase HRI) in a dose dependent manner. In addition, novobiocin-induced inhibition of the Hsp90/Cdc37-dependent activation of HRI's autokinase activity correlated well with its ability to disrupt the interaction of Hsp90 and Cdc37 with newly synthesized HRI. The concentration range required to inhibit HRI's Hsp90-dependent transformation was similar to the range of novobiocin concentrations that Kanelakis and co-workers found was required to inhibit Hsp90-dependent restoration of steroid hormone binding to the glucocorticoid receptor in vitro using purified proteins (136).

The mechanism by which novobiocin inhibits Hsp90 function appears to be distinct from that of geldanamycin and molybdate, as novobiocin has effects distinct from those of geldanamycin and molybdate both on the basal interaction of co-chaperones with Hsp90, and on the interactions of Hsp90/Cdc37 and co-chaperones with the Hsp90 client kinase, HRI. Of further interest is the observation that the binding of novobiocin to Hsp90's C-terminal domain decreased the interaction of Hsp90 with co-chaperones containing TPR domains, which interact with Hsp90's C-terminal EEVD motif. This suggests that novobiocin-binding may alter the conformation of Hsp90's C-terminus leading to the dissociation of TPR containing co-chaperones. Of note is that while novobiocin markedly reduced or eliminated the interaction of Hsp90 with PP5 and FKBP52, it had less of an inhibitory effect on the basal interaction of HOP with Hsp90. This difference probably reflects the fact that HOP interacts with Hsp90 early in its ATP cycle at a point when the C-terminal nucleotide binding site may be inaccessible, or more

likely in a low affinity conformation. In contrast, FKBP52 and PP5 are present in late complexes in which the C-terminal nucleotide-binding site would be open and readily accessible for novobiocin binding. These results are also consistent with the observations that the binding of HOP *versus* PP5 or FKBP52 to Hsp90 is mutually exclusive, and that HOP interacts with Hsp90 when its N-terminal domain is in its "open", possibly nucleotide-free conformation (29).

Novobiocin had effects on the interactions of Hsp90 with co-chaperones and client kinase that were dominant to the effects of molybdate. Novobiocin altered the co-chaperone composition of basal Hsp90 complexes, destabilized the binding of Hsp90/Cdc37 to HRI, and changed the interactions of co-chaperone with HRI even when lysate was pre-incubated with molybdate. The dominance of the effect of novobiocin over molybdate and the ability of novobiocin to quantitatively dissociate Hsp90/Cdc37-kinase complexes suggests that novobiocin may bind to Hsp90 subsequent to molybdate and induce kinase release. The notion that novobiocin may bind to Hsp90 after molybdate is supported by observations which suggest that the interaction of novobiocin with Hsp90 occurs after the formation of "late" Hsp90-client complexes. Geldanamycin induces accumulation of intermediate Hsp90 complexes that contain Hsc70 and weakly bound client, while molybdate stabilizes Hsp90/Cdc37-client complexes that contain "late" components of the Hsp90 machine (119, 120). Nucleotide-modulated switching of Hsp90's conformation establishes Hsp90's and Cdc37's high-affinity salt-stabile binding to kinase (41, 141). Molybdate and p23 stabilize Hsp90 binding to client, and the binding of molybdate and p23 to Hsp90 in the absence of client is thought to induce a similar

Hsp90 conformation. Thus, the observation that p23, FKBP52 and PP5 ("late" co-chaperone components of the Hsp90 machine) remain associated with HRI client after novobiocin-induced dissociation of Hsp90 and Cdc37 from HRI supports the hypothesis that novobiocin binds Hsp90 subsequent to the formation of late Hsp90/co-chaperone client complexes. This notion would be consistent with the observed absence of Hsc70 (a component of intermediate Hsp90 heterocomplexes) and p23 (a component of late Hsp90 heterocomplexes) from basal Hsp90 complexes isolated from lysate incubated in the presence of novobiocin [Fig. 6 and (26)]. Again, the dominance of the effects of novobiocin over molybdate-induced stabilization of Hsp90-client complexes suggests that the novobiocin-bound conformation of Hsp90 may be responsible for client release.

The observation that p23, FKBP52, and PP5 interact directly with HRI after novobiocin-induced dissociation of Hsp90 and Cdc37 suggests that these co-chaperones may play a role in chaperoning client folding or communicating changes in client conformation in some manner to Hsp90. While we had previously demonstrated a direct interaction of PP5 with HRI (129), past data had not allowed us to deduce whether FKBP52 or p23 had any direct interaction with HRI or whether they were present in Hsp90-kinase complexes simply due to their binding affinity for Hsp90 (141). The direct interaction of p23 and FKBP52 with HRI is consistent with the chaperone activity that these two proteins display in assays *in vitro* (107, 148, 149), and the observation that p23 is present in stable complexes with some client proteins, presumably after their dissociation from Hsp90 (81, 150, 151). This observation also invites the speculation that the interaction of these proteins with HRI may play a role in the reassembly of Hsp90 complexes with immature HRI, as reiterative cycles of Hsp90 action are required for kinase maturation (42, 141). Furthermore, the interactions could play additional roles in

regulating HRI function, such as the proposed role of FKBP52 in intracellular localization of Hsp90-dependent clients (13, 152). This latter possibility is of interest, as novobiocin-treatment appears to enhance the interaction of FKBP52 with HRI, and some "matured and activated" HRI, which exhibits a slower electrophoretic mobility, was co-immunoadsorbed with FKBP52 from heme-deficient lysate (Fig. 7C).

It has been suggested, however, that novobiocin might affect the function of the Hsp90 chaperone machine by affecting Hsc70 as well (136). While our data cannot conclusively rule out this possibility, the work presented here (which is discussed below) clearly demonstrates that novobiocin binds Hsp90 and directly alters its structure and dynamic, such that at any given time basal heterocomplexes containing Hsp90 can be in the process of assembly or disassembly. Thus, we favor a model in which novobiocin compromises Hsc70's function indirectly through altering Hsp90's structure at a specific point in its reaction cycle and subsequently compromising Hsc70's ability to interact productively with the Hsp90 chaperone machine.

The notion that novobiocin can affect the function of co-chaperone components 'indirectly' through its effects on Hsp90 function is supported by additional observations. Addition of molybdate reproducibly caused an approximate 30% decrease in the interaction of HOP with Hsp90, and a significant decrease in the binding of Hsc70. The presence of molybdate would stabilize "late" complexes formed from intermediate Hsp90/HOP/Hsc70 heterocomplexes and cryptic clients present in the reticulocyte lysate. These complexes would lack Hsc70 and HOP, but be sensitive to novobiocin-induced dissociation of cryptic substrate. On the other hand, Hsp90/HOP complexes are thought

to be nucleotide-free prior to the association of Hsc70 containing bound client (30, 71, 131). As such, Hsp90's C-terminal nucleotide-binding site in these complexes would be in its low affinity conformation, and only be sensitive to novobiocin at very high concentrations, as we have observed. In addition, since nucleotide bound to Hsp90's N-terminus appears to be required for molybdate-induced effect on Hsp90's conformation(77, 118), the C-terminal nucleotide binding site of nucleotide-free Hsp90/HOP would remain in its low affinity conformation, which is consistent with the observation that high concentrations of novobiocin are still required to cause a decrease in Hsp90/HOP complexes in the presence of molybdate. The observation that the intensity of the 78 kDa N-terminal tryptic fragment generated in the presence of molybdate and 5 mM novobiocin is only reduced by approximately 80% (Fig. 8B, discussed below), suggests that about 20% of the Hsp90 molecules present in reticulocyte lysate may be in a nucleotide-free form under our assay conditions. Approximately 30% of the Hsp90 in reticulocyte lysate appears to be bound to HOP (*1*). Consistent with its unique effects on the interactions of Hsp90 with client and co-chaperones, novobiocin also had distinct effects on the conformation of Hsp90 as assessed by proteolytic fingerprinting of Hsp90 *in situ*. Fingerprinting of Hsp90 indicated that addition of novobiocin had little effect on the default conformation of Hsp90 at concentrations (1-2 mM) that significantly inhibited HRI transformation and induced the dissociation of Hsp90 and Cdc37 from newly synthesized HRI (Fig. 4). Similarly, 1-2 mM novobiocin had little effect on the proteolytic fingerprint of Hsp90 generated in the presence of geldanamycin. However, very high concentrations of novobiocin (10 to 20 mM) were found to alter the proteolytic fingerprint of Hsp90 both in the presence[2] and absence of geldanamycin. Results from other laboratories indicates that occupancy of the N-

terminal ATP binding site is required for the accessibility of the second C-terminal ATP binding site to novobiocin (26, 27). Our results suggest that the default and geldanamycin-bound conformations of Hsp90 maintain the C-terminal nucleotide-binding site in a low-affinity conformation, but that novobiocin induced switching of Hsp90's conformation can occur in the presence of a sufficiently high concentration of novobiocin. The conformational change induced by the binding of novobiocin appears to be highly cooperative with a Hill coefficient of 1.7, suggesting that both novobiocin-binding sites within an Hsp90 dimer must be occupied for the conformational switching to occur.

The molybdate-induced switching of Hsp90's conformation dramatically enhances the binding affinity of Hsp90's C-terminal nucleotide binding site for ligand. This hypothesis is supported by the observation that 2 mM novobiocin has a dramatic effect on the proteolytic fingerprint of Hsp90 when added to reticulocyte lysate in the presence, but not in the absence of molybdate. The interaction of Hsp90/Cdc37 with newly synthesized HRI was also markedly reduced both in the presence or absence of molybdate when 2 mM novobiocin was added to reticulocyte lysate (Fig. 5 and 7). Furthermore, 2 mM novobiocin blocked the Hsp90/Cdc37-dependent transformation of HRI in heme-deficient lysate (Fig. 5B, 6B, and 7B). Binding of molybdate to Hsp90 stabilizes Hsp90 in a conformation that mimics the properties of salt-stabile high-affinity complexes formed between Hsp90/Cdc37 and bound client following nucleotide modulated conformation switching of Hsp90 (125, 141). Thus, ATP-induced "clamping" of Hsp90's N-terminal domain and the subsequent hydrolysis of this ATP may markedly enhance to accessibility or the affinity of Hsp90's C-terminal nucleotide binding site. Since molybdate markedly enhanced the binding affinity of Hsp90 for novobiocin, it is

likely that the release of bound phosphate [of which molybdate has been proposed to be a high affinity binding mimetic (77)] is an important step kinetically in the regulation of the Hsp90 reaction cycle (125). Past work identifying the location of the major sites in Hsp90 that are cleaved by trypsin (145, 146) now allows us to accurately identify the sites within Hsp90 whose cleavage is altered upon the binding of molybdate and novobiocin (Fig. 11). Relative to the amino acid sequence of human Hsp90α, Hsp90 has major trypsin cleavage sites at residues 288, 399 and 614/619, and minor cleavage sites near residues 228, 250 and 573 (145, 146). The sites at residues 228, 250 and 288 represent cleavage of Hsp90 near the N-terminus, the middle and the C-terminus of the highly charged linker region that connects the N-terminal nucleotide binding domain and the middle domain of Hsp90 [Fig. 3A, 11, and (145, 146)]. K573 is located at the C-terminal end of a highly charged sequence that is present between the middle and C-terminal domains of Hsp90 (145). The site at 399 is within the region of Hsp90 that has been proposed to interact with (27) and catalyze the hydrolysis of (31) the γ-phosphate of ATP bound to the N-terminal domain of Hsp90, as well as being adjacent to the site that has been proposed to be the client binding pocket (31). The site at residue 614/619 is adjacent to the region. that binds novobiocin (26, 27), and the site at 573 is just upstream from this region.

Consistent with its ability to inhibit Hsp90 proteolysis at residue 399, molybdate is thought to act as a phosphate mimetic which binds to the site occupied by the γ-phosphate of the ATP bound to the N-terminal domain of Hsp90 after its hydrolysis (77). Our previous work also indicates that molybdate reduces cleavage of Hsp90 at residue

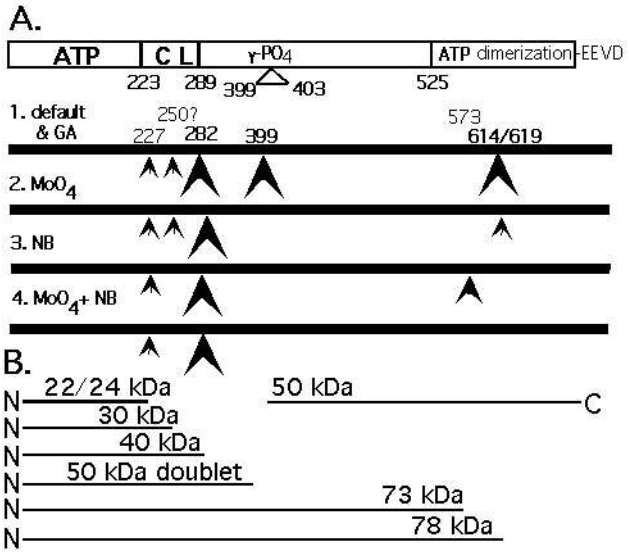

Figure 11. Summary of effects of pharmacological agents on the proteolytic fingerprint of Hsp90. (A) 1) Trypsin cleavage sites of Hsp90 in its default and geldanamycin-bound conformations. 2) Trypsin cleavage sites of Hsp90 in its molybdate-bound conformation. 3) Trypsin cleavage sites of Hsp90 in its novobiocin-bound conformation. 4) Trypsin cleavage sites of Hsp90 in the presence of molybdate and novobiocin. Large arrow, major cleavage site; small arrow, minor cleavage site; presence or absence of arrow, or altered size indicates change in susceptibility to cleavage by trypsin. (B) Origin of N- and C-terminal Hsp90 fragments. Note that N-terminal fragments containing the charge linker region (CL) have abnormal electrophoretic mobility. ATP, nucleotide binding sites; γ-PO_4, putative site of interaction of the γ-phosphate of the ATP bound to Hsp90's N-terminus.

614/619 (125). Our current work demonstrates that novobiocin (and MgATP) quantitatively protects Hsp90 from cleavage at the 614/619 site independent of molybdate. Thus, the ability of molybdate to retard cleavage at this site may represent molybdate's ability to enhance interaction of endogenous nucleotide with the C-terminal nucleotide-binding site of Hsp90. In addition, the observation that novobiocin and MgATP protect Hsp90-CT from proteolytic cleavage indicates that the binding of novobiocin or nucleotide to the C-terminal site is directly responsible for the altered Hsp90 conformation, and that the protection from proteolysis is not caused by altering the binding of a co-chaperone to the site that would render the site inaccessible to protease. However, it cannot be excluded at this time that the changes in protease sensitivity of Hsp90 at other sites might be due to alterations in the interactions of co-chaperones with Hsp90 as opposed to direct changes in structure. Because dissociation of client kinase from Hsp90/Cdc37 complexes in all likelihood requires the "unclamping" of the N-terminal nucleotide binding domains within the Hsp90 dimer, the data suggest that the binding of novobiocin to Hsp90 may induce this event. Since the N-terminal domain of Hsp90 does not bind novobiocin (26, 27), occupancy of the C-terminal nucleotide-binding site of Hsp90 by novobiocin would need to be communicated to Hsp90's N-terminal domain through its effects on the conformation of Hsp90 at its C-terminus (26, 27). This proposed communication between the N- and C-terminal domains of Hsp90 is consistent with several observations: 1) occupancy of the N-terminal ATP binding site of Hsp90 is required for the C-terminal nucleotide binding site to become accessible (27, 124); 2) binding of novobiocin to the C-terminal nucleotide binding site disrupts nucleotide binding at the N-terminus (27); 3) sequences in the C-terminus of Hsp90 regulate the hydrolysis of ATP bound to the N-terminal domain of Hsp90 (33, 128),

which is required for the ATP-dependent formation of stable Hsp90-client complexes containing p23 (77, 116, 118, 128, 131); and 4) our proteolytic fingerprints indicate that novobiocin causes structural changes (and/or altered co-chaperone/client interactions) in the C-terminal domain, the middle domain, and the linker region between the N-terminal and middle domains of Hsp90. *In toto*, the data presented in this paper suggest that the binding of novobiocin to the C-terminal nucleotide-binding site of Hsp90 stimulates the dissociation of Hsp90 from client protein. Since the binding of novobiocin to Hsp90 inhibits binding of nucleotide to the N-terminal domain of Hsp90 (26, 27), and N-terminally bound nucleotide is required to stabilize Hsp90 in its "closed", high-affinity client binding conformation (24, 116, 118), we propose that novobiocin binding induces the dissociation of nucleotide from the N-terminal domain of Hsp90, the destabilization of p23 binding and kinase release. The questions of whether this model will apply to other or all Hsp90 client targets, as well as what stimulates the recycling of Hsp90 from its novobiocin bound state remain to be explored.

CHAPTER III

Hsp90 Functions to Balance the Phosphorylation State of Akt during C2C12 Myoblast Differentiation: a potential anti-apoptotic effect of Hsp90 inhibitors

Abstract

Hsp90 function is essential for the regulation of a myriad of signal transduction cascades that control all facets of a cell's physiology. Akt (PKB) is an Hsp90-dependent serine-threonine kinase that plays critical roles in the regulation of muscle cell physiology, including roles in the regulation of muscle differentiation and anti-apoptotic responses that modulate cell survival. In this report, we have examined the role of Hsp90 in regulating the activity of Akt in differentiating C2C12 myoblasts. While long-term treatment of differentiating C2C12 cells with the Hsp90 inhibitor geldanamycin led to the depletion of cellular Akt levels, pulse-chase analysis indicated that geldanamycin primarily enhanced the turnover rate of newly synthesized Akt. Hsp90 maintained an interaction with mature Akt, while Cdc37, Hsp90's kinase-specific co-chaperone, was lost from the chaperone complex upon Akt maturation. Geldanamycin partially disrupted the interaction of Cdc37 with Akt, but had a much less significant effect on the interaction of Hsp90 with Akt. Surprisingly, short-term treatment of differentiating C2C12 with geldanamycin increased the phosphorylation of Akt on Ser^{473}, an effect mimicked by treatment of C2C12 cells with okadaic acid or the Hsp90 inhibitor novobiocin. Furthermore, Akt was found to interact directly with PP2Ac in C2C12 cells,

and this interaction was not disrupted by geldanamycin. Our findings indicate that Hsp90 functions to modulate PP2A action on Akt during C2C12 myoblast differentiation. While previous studies have indicated that PP2A plays a role in regulating Akt activation, our data suggests that Hsp90 modulates the ability of Akt to be dephosphorylated when it is bound in an Akt-PP2A complex.

Introduction

The Akt (PKB) serine-threonine protein kinase plays amajor role in regulating signal transduction pathways that are activated by cellular growth factors [reviewed in (153)]. Akt function is integrated with pathways that control cell proliferation, size, differentiation and viability. Since altered Akt function has been associated with the etiology of a number diseases states, including cancer and diabetes, a better understanding of Akt regulation is needed to develop adequate therapies for treatment of diseases associated with its dysregulation (153).

Akt plays important roles in regulating muscle cell physiology [reviewed in (154-159)]. Stimulation of Akt has hypertrophic effects on skeletal and cardiac muscle (160-163). In addition to playing a positive role in stimulating myoblast proliferation, Akt function is required for muscle cell differentiation (164-168). Subsequently, Akt has positive effects on cell growth and viability of myotubules (111, 153, 155, 157-159). Thus, Akt function contributes to the regulation of the number, size and survival of mature muscle cells.

Regulation of Akt activity is complex and not yet thoroughly understood [reviewed in (153, 159)]. Akt is activated upon stimulation of growth factor and integrin

receptors. Akt activation in response to growth factors occurs primarily through receptor-mediated activation of PI3-kinase. Direct binding of the N-terminal pleckstrin homology domain of Akt to PI3-kinase generated phospholipids causes the translocation of Akt to the plasma membrane and leads to Akt activation via its phosphorylation by PDK1 (Thr^{308}) and PDK2 (Ser^{473}). Additional protein kinases (153), such as integrin-linked kinase-1 (169), also modulate Akt activity through their ability to phosphorylate Akt on Ser^{473}. Once activated, maintenance of Thr^{308} phosphorylation does not appear to be required for Akt to remain active (170), and Akt may be capable of maintaining its activity through its autophosphorylation at Ser^{473} (171).

Akt activity is also modulated through regulation of its dephosphorylation (137, 172-179). Protein phosphatase 2Ac is the principal phosphatase that has been implicated in regulation ot Akt activity (137, 172, 174-176, 178, 179). The best characterized example of phosphatase-modulated regulation of Akt is the ability of integrin-$\alpha 2\beta 1$ receptor stimulation to activate PP2Ac and induce the dephosphorylation of Akt (179).

The molecular chaperone Hsp90, together with its co-chaperone partner Cdc37, functions to facilitate the folding and activation of numerous protein kinases that regulate signal transduction pathways in cells [reviewed in (13, 29, 35, 101, 110, 180)]. Hsp90 contains two nucleotide binding sites and it carries out its function through the binding and hydrolysis of ATP, which is modulated in part through the interactions of Hsp90 with its co-chaperone partners and its target clientele (13, 29, 101, 181-183). Geldanamycin and novobiocin inhibit Hsp90 function by binding to nucleotide-binding sites located in the N-terminal and C-terminal domains of Hsp90, respectively, and thus, blocking Hsp90's ATP-driven reaction cycle.

An interaction of Hsp90 (48, 99) and Cdc37 (48) with endogenous Akt has been documented in cancer cell lines, and Akt function is known to be dependent upon Hsp90 and Cdc37 (48, 99, 184, 185). Treatment of cultured cells with geldanamycin-derivative, 17-allylaminogeldanamycin (17-AAG) leads to a depletion of Akt protein (48). Treatment of cells with 17-AAG did not appear to disrupt the interaction of Cdc37 with Akt, and the 17-AAG-induced decrease in the interaction of Cdc37 with Akt occurred in parallel with the loss of Akt protein from cells (48). However, no direct data documenting the effect of geldanamycin or its derivatives on the interaction of Hsp90 with endogenous cellular Akt has been presented. In addition, HA-tagged Akt was found in complexes with Hsp90 only when Cdc37 was co-transfected and overexpressed (48).

Most studies attribute geldanamycin-induced loss of Hsp90-dependent protein clientele, including Akt (48), to its ability to disrupt the productive interaction of Hsp90 with its client targets, leading to the destabilization of Hsp90 clients, their ubiquitination, and subsequent proteolysis by the proteasome (110, 180). In contrast, Tsuruo and co-workers have suggested that Hsp90 dependence of Akt activity is the result of Hsp90 protecting Akt from dephosphorylation by PP2A (99), in conjunction with the dependence of the Akt-activating kinase, PDK1 on Hsp90 function (185). However, their experimental paradigms utilized Akt overexpressed in cells by transfection, which does not allow one to assess the effect of prolonged Hsp90-inhibition (24 h) on the activity, stability or turnover of previously matured Akt molecules (99, 185). Thus, the effect of Hsp90 inhibition on the interaction of Hsp90 and Cdc37 with endogenous cellular Akt, and its maturation and stability warrants further investigation

The process of skeletal muscle differentiation is regulated by contact-inhibition of cell growth, cell growth factors and cytokines (160, 166, 181-183, 186). C2C12 myoblasts proliferate in medium containing serum with high growth factor concentrations (fetal serum), but are induced to differentiate upon incubation of confluent cells in medium containing low growth factor concentrations (horse serum). During this processes, myoblasts withdraw from the cell cycle, express a number of muscle specific genes, and fuse into multinucleated myotubes. Akt has been shown to play a critical role in regulating the differentiation and survival C2C12 myoblasts/myotubules (166, 186, 187).

In this report, we have examined the effects of inhibition of Hsp90 on Akt protein stability and activity in differentiating C2C12 myoblasts. Contrary to conclusions drawn in previous reports utilizing cancer cell lines (48, 99), geldanamycin primarily destabilized newly synthesized Akt, while only marginally affecting the stability of mature Akt kinase molecules. Of particular interest was the observation that short-term treatment with Hsp90 inhibitors enhanced Akt activation, apparently through their ability to disrupt the ability of PP2Ac to dephosphorylate Akt present in Akt/Hsp90 complexes.

Experimental Procedures

Cell culture. C2C12 myoblast (from ATCC) cells were grown in DMEM (BioWhitaker) containing 10% fetal calf serum (Hyclone) and streptomycin/penicillin (100 units/mL, Sigma) at 37ºC, in the presence of 5% CO_2 and 95% air. For induction of differentiation, confluent cells were maintained in DMEM containing 5% equine serum (Hyclone) (differentiation media, DM).

Analysis of effects of pharmacological treatment of C2C12 cells. Confluent cells were maintained in DM for 40 h, and then treated with the given pharmacological agent or solvent control for 0 to 24 h as indicated in the legend to the figures. After washing the cells briefly with phosphate-buffered saline (PBS), the cells were lysed with SDS sample buffer. Samples were boiled in a water bath for 5 min, separated by SDS-PAGE, and electro-transferred to PVDF membrane (Bio-Rad). Proteins were analyzed by western blotting with anti- Akt, anti-phospho-Ser^{473} or Thr^{308} specific Akt (Cell Signaling), anti-GSK3β, or anti-Hsp90 antibodies.

Pulse-chase analysis of Akt expression. Confluent cells were maintained in DM for 40 h, followed by metabolic labeling with [^{35}S]methionine (100 µCi/mL, PerkinElmer). For experiments analyzing the turnover of newly synthesized Akt kinase, geldanamycin (5 µg/mL) or vehicle control (DMSO) was added to the DM during the 30 min labeling period with [^{35}S]methionine. After 30 min, the cells were washed and maintained in fresh DM containing geldanamycin (5 µg/mL:9 µM) for 0 to 4 h. For experiments analyzing the turnover of mature kinase, cells were incubated with [^{35}S]methionine for 30 min. The cells were then washed and maintained in fresh DM medium for 3h. Geldanamycin (5 µg/mL) or DMSO was then added for 0 to 24 h. At the time points indicated in the figure legends, cells were washed with phosphate-buffered saline (PBS), and then lysed with RIPA lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 0.25% deoxycholic acid, 1% SDS, 2mM EDTA, 1mM EGTA and protease inhibitor cocktail (Sigma)). Samples were clarified by centrifugation for 15 min at maximum speed in a microcentrifuge (~12000 rpm), and Akt was immunoadsorbed with anti-Akt antibody (Cell Signaling) at 4ºC for 2 to 3 h. Samples were washed 3 times with

lysis buffer, boiled in SDS sample buffer and analyzed by SDS-PAGE on 8% gels. Proteins were electro-transferred to PVDF membrane, and total Akt in each sample was determined by western blotting with anti-Akt antibody. [^{35}S]-labeled Akt was visualized by autoradiography.

Co-immunoadsorption of Akt with Hsp90/Cdc37 or PP2Ac. Confluent cells were maintained for 40 h as described previously. Cells were treated with geldanamycin (5 µg/mL) or DMSO for 30 min and then lysed with lysis buffer (50 mM HEPES pH 7.5, 100 mM NaCl, 0.1% deoxycholic acid, 0.1% Triton X-100, 20 mM sodium molybdate, 50 mM NaF, 2 mM EDTA, 1 mM EGTA, and protease inhibitor cocktail (Sigma)). Cell lysate was clarified by centrifugation for 20 min at ~ 12000 rpm. Akt or PP2A was immunoadsorbed with anti-Akt or anti-PP2Ac (Upstate Biotechnology) antibody at 4º C for 2 to 3 h. Samples were washed 4 times with washing buffer (10 mM PIPES pH 7.2, 150 mM NaCl) and then analyzed by western blotting with anti-Hsp90, anti-Cdc37, anti-Akt or anti-PP2Ac as indicated in the figure legends.

To assay PP2Ac activity present in cell extracts, immunoadsorbed PP2Ac was washed 2 times with wash buffer containing 20 mM NaF and then 2 times with phosphatase assay buffer (50 mM Tris-HCl pH 7.0, 100 µM $CaCl_2$). Samples were assayed as described as protocol of the manufacturer's PP2A assay kit (Upstate Biotechnology). To determine the effect of geldanamycin on PP2A activity in vitro, samples were mixed with phosphatase substrates in the presence of 10 µg/mL geldanamycin or DMSO and then incubated at 30º C for 10 min. The assay was terminated by addition of a Malachite Green mixture solution and samples were analyzed using a Microplate autoreader (Bio-TEK Instruments) at 660 nm.

In vitro transcription/translation of Akt in rabbit reticulocyte lysate. The coding sequence of Akt was excised from a plasmid containing HA-tagged mouse Akt1 (kindly provided by Dr. Phillip Dennis, NCI) using HindIII and XbaI restriction enzyme sites, and cloned into pSP64T, as described previously (140), to construct pSP64T encoding Akt with a His_6-tag at its N-terminus. His-tagged Akt was synthesized in rabbit reticulocyte lysate (Promega) by coupled transcription/ translation (28, 138, 140) in the presence of (5 µg/mL) geldanamycin or DMSO and then matured for the times indicated in the figure legends. Protein synthesis was determined to terminate at approximately 25 min in this lot of lysate. Akt was immunoadsorbed with anti-His_5 monoclonal antibody (Qiagen) bound to anti-mouse agarose resin at 4ºC for 1 to 2 h as previously described (28, 138, 140). Samples were washed with 4 times with wash buffer and analyzed by SDS-PAGE on 8% gels. [^{35}S]-labeled Akt was visualized by autoradiography and co-adsorbed Hsp90 and Cdc37 were detected by western blotting.

Results

Effects of geldanamycin on Akt expression in differentiating C2C12 myoblasts. While the effect of Hsp90 inhibition on the fate of Hsp90-dependent protein kinases has been extensively studied in proliferating cancer cells, few studies have examined the effects of geldanamycin on cells that have withdrawn from the cells cycle. Upon reaching confluence and withdrawal of serum containing high levels of growth factors, C2C12 myoblasts enter into G_0, and proceed to differentiate and fuse into multinucleated myotubules. Akt, a well-characterized Hsp90-dependent protein kinase (48, 99, 173), is known to play an essential role in regulating C2C12 myoblast differentiation (165, 166,

168). Therefore, we examined the effect of geldanamycin on Akt expression in myogenic C2C12 cells during their differentiation.

Two days after induction of differentiation, C2C12 cells were treated with geldanamycin for 0 to 24 h. Western blot analysis of cell extracts indicated that the level of Akt was reduced gradually over the course of the 24 h geldanamycin treatment compared to control cells, while the level of Hsp90 expression was maintained (Fig 12A). Addition of the protease inhibitor ALLN or the proteasome-specific inhibitor lactacystin to geldanamycin-treated cells protected Akt from geldanamycin-induced turnover (Fig. 12B). The partial reduction of Akt observed in the presence of geldanamycin and protease inhibitors was likely due to accumulation of polyubiquitinated Akt species which would not have been detected under our western blot conditions. These results suggest that Akt is degraded via a proteasome-dependent pathway in geldanamycin-treated cells, which is consistent with the effects of geldanamycin on Akt levels reported in a previous study using a different cell line (48).

Subsequently, pulse-chase analysis was utilized to determine the mechanism by which geldanamycin-treatment led to the depletion of Akt levels in differentiating C2C12 cells. Newly synthesized Akt was pulse-labeled with [^{35}S]-Met in the presence or absence of geldanamycin for 30 min. Cells were then incubated in fresh media for 0 to 4 h in the presence or absence of geldanamycin. The turnover rate of the newly synthesized [^{35}S]-labeled Akt was determined by immunoadsorption of Akt from cell extracts, SDS-PAGE and autoradiography (Fig. 12C). Newly synthesized Akt turned over rapidly within 1 h of “chasing” the cells into fresh media. At the 0 h time point, the

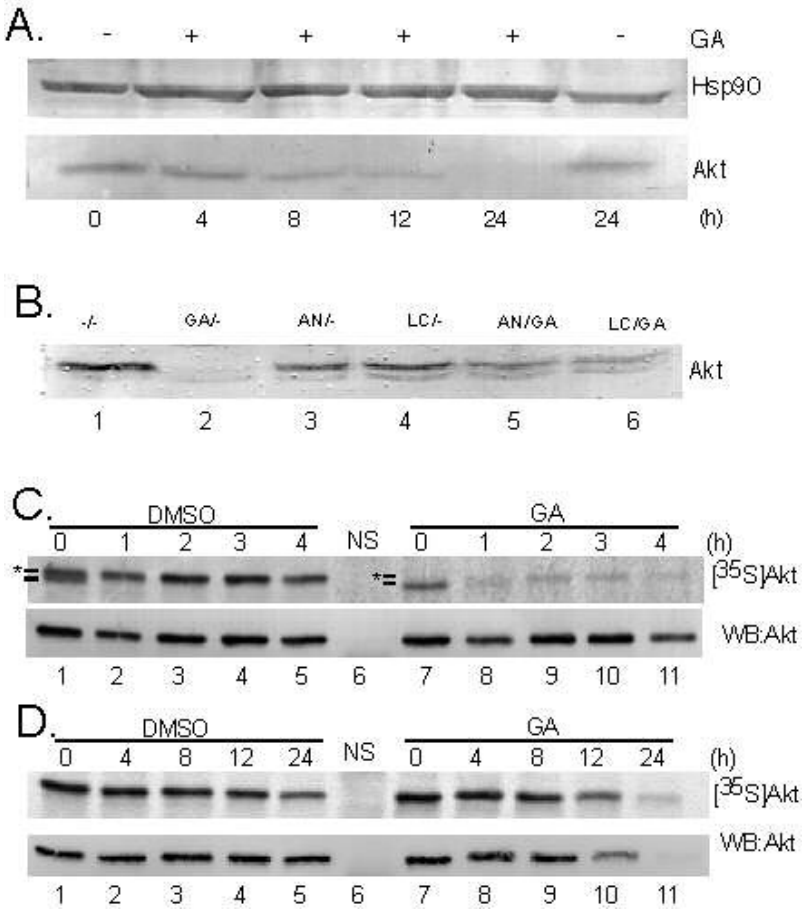

Figure 12. Effects of geldanamycin on Akt protein levels and turnover. (A) Confluent C2C12 cells were maintained in differentiation medium (DM) for 40 hrs and then treated with geldanamycin (+) or DMSO (-) for 0 to 24 h as indicated. (B) Cells were treated with DMSO (-/-, lane 1), 5 µg/mL:9 µM geldanamycin (GA/-, lane 2), 10 µM ALLN (AN/-, lane 3), 10 µM lactacystin (LC/-, lane 4), 10 µM ALLN plus 5 µg/mL:9 µM geldanamycin (AN/GA, lane 5) or 10 µM lactacystin plus 5 µg/mL geldanamycin (LC/GA, lane 6) for 24 hrs. Cells were washed and lysed directly with SDS buffer, and analyzed by SDS-PAGE and western blotting with anti-Hsp90 and anti-Akt antibodies. Pulse-chase experiments to determine the turnover rates of newly synthesized (C) or mature (D) [^{35}S]-Akt were carried out as described under "Experimental Procedures". Briefly, Cells were pulsed labeled with [^{35}S]Met for 30 min. DMSO (lanes 1-5) or 5 µg/mL geldanamycin (GA; lanes 7-11) were present during the labeling period and chase (C), or added 3 h after the initiation of the chase (D). Akt was immunoadsorbed with anti-Akt agarose resin from cell extracts prepared at the times (h) indicated after the initiation of the chase. Akt was detected by autoradiography ([^{35}S]Akt) or western blotting (WB:Akt) after analysis of samples by SDS-PAGE. NS: non-immune IgG as a control for non-specific binding (C and D, lane 6).

[^{35}S]-Akt in extracts from control cells appeared as a doublet that shifted to a single band with slower electrophoretic mobility at later time points. In contrast, most of the [^{35}S]-Akt present in extracts from geldanamycin treated cells co-migrated with the band from the control cell extracts that had the faster electrophoretic mobility. Furthermore, the minor amount [^{35}S]-Akt that was present in extracts from geldanamycin-treated cells that had slower electrophoretic mobility did not turnover during the time course of the experiment. These observations suggested that Akt was undergoing phosphorylation (commonly associated with acquisition of slower electrophoretic mobility (48, 138) during its maturation, and that the little Akt that had "matured" in geldanamycin-treated cells had attained a stable conformation.

Work with other cell lines has suggested that geldanamycin-induced depletion of Akt protein (48, 188, 189) is due to accelerated turnover of Akt (48). To test this hypothesis, Akt was pulsed labeled in differentiating C2C12 cells for 30 min, and then matured for 3 h prior to exposure of the cells to geldanamycin. In three independent experiments, geldanamycin-treatment caused little significant change in the turnover rate of Akt during the first 8 h of exposure of the cells to the drug (Fig. 12D). However, between 8 and 12 h of treatment of the cells with geldanamycin, the turnover of Akt was clearly accelerated relative to the control, and near complete turnover of the Akt was noted after 24 h of treatment. Thus, while the data clearly indicate that newly synthesized Akt requires geldanamycin-inhibitable Hsp90 function for its folding and stability, the requirement of mature Akt for Hsp90 function is more subtle, as little impact on Akt protein levels were noted for the first 8 h of incubation with geldanamycin.

Effect of geldanamycin on the interaction of Hsp90 and Cdc37 with Akt. In previous studies, we have utilized coupled transcription/ translation in rabbit reticulcyte lysate to model interaction of Hsp90 and Cdc37 with the Src-family tyrosine kinases, Lck and Hck, and the heme-regulated eIF2α kinase (HRI) (138-141, 190-192). Hsp90 and Cdc37 were observed to dissociate from Hsp90/Cdc37-kinase complexes as newly synthesized kinases matured into their active conformations (41, 138, 139, 141, 190). Furthermore, these studies indicated that geldanamycin-inhibitable Hsp90 function was obligate for these protein kinases to attain an active conformation, and that geldanamycin-treatment prevented the formation of Hsp90-kinase complexes that were stable to washing in buffers containing high salt concentrations(41, 45, 138, 139, 141, 190). In addition, geldanamycin blocked the interaction of Cdc37 with the salt-labile Hsp90-kinase complexes that form in its presence in rabbit reticulocyte lysate (41, 45, 129, 141).

Therefore, His-tagged [^{35}S]-Akt was synthesized and matured in reticulocyte lysate. Both Hsp90 and Cdc37 were co-immunoadsorbed with His-tagged Akt after its synthesis was terminated at 25 min (Fig. 13B, lane 1). Relative to the amount of Cdc37 bound to Akt immediately after termination of its synthesis, the interaction of Akt with Cdc37 was reduced by 70 % and >95% after maturation of the kinase for 60 and 90 min, respectively. In contrast, interaction of Akt with Hsp90 was reduced by only 25% and 40% after the 60 and 90 min maturation, respectively, after normalization to the amount of His-tagged [^{35}S]-Akt immunoadsorbed from the reticulocyte lysate (Fig. 13B). Thus, a stable interaction of Hsp90 with a population of Akt molecules appears to be maintained after a maturation period that results in nearly the complete loss of associated Cdc37.

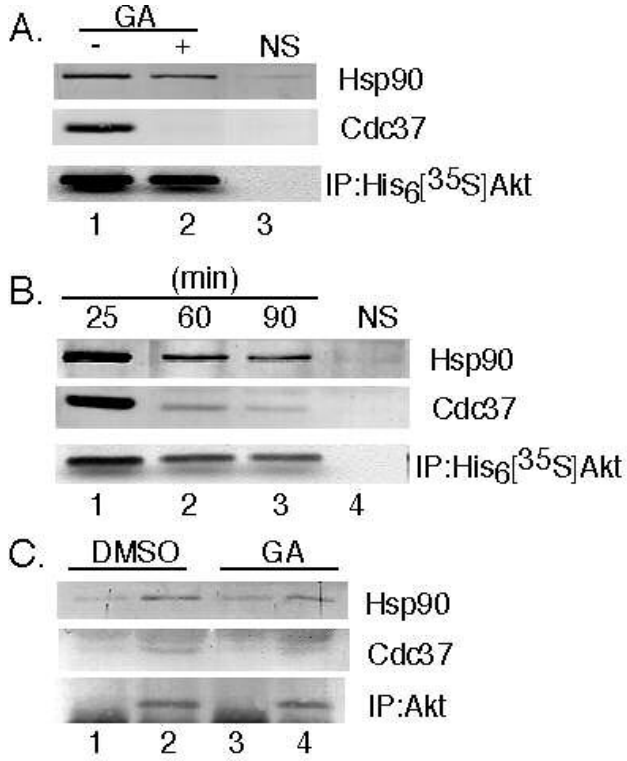

Figure 13. Effects of geldanamycin on the interaction of Akt with Hsp90 and Cdc37. (A) [^{35}S]-labeled His-tagged Akt was synthesized for 30 min in the presence of DMSO (-, lane 1) or 10 µg/mL geldanamycin (GA) (+, lane 2) in reticulocyte lysate. The samples were immunoadsorbed with anti-His antibody and analyzed by western blotting with anti-Hsp90 (Hsp90) and anti-Cdc37 (Cdc37) antibodies and autoradiography (IP: His$_6$[^{35}S]Akt). (B) [^{35}S]-labeled His-tagged Akt was synthesized for 25 min (lane 1) and then matured for an additional 35 (lane 2, 60) or 65 (lane 3, 90) min as indicated. [^{35}S]-labeled His-tagged Akt was immunoadsorbed and analyzed as described above. NS (A. lane 3 & B. lane 4): Analysis of sample lacking template coding for His-tagged Akt as a control for nonspecific binding of Hsp90 and Cdc37 to the immune resin. (C) C2C12 cells were maintained in DM for 40 hrs, and then treated with DMSO (lane 1 and 2) or 5 µg/mL geldanamycin (GA; lane 3 and 4) for 3 h. Cell lysates were prepared as described under "Experimental Procedures". Akt was immunoadsorbed with anti-Akt antibody resin (lanes 2 and 4) or a nonspecific IgG antibody resin (lanes 1 and 3), and Hsp90, Cdc37 and Akt were detected by western blotting.

Subsequently, the effect of geldanamycin on the interaction of Hsp90 and Cdc37 with the newly synthesized Akt was assessed (Fig. 13A). Western blot analysis of samples prepared by immunoadsorption with anti-His tag antibodies using buffers with a moderate salt concentration (150 mM NaCl) indicated that Hsp90 and Cdc37 co-adsorbed with newly synthesized Akt, and that geldanamycin quantitatively blocked the interaction of Cdc37 with Akt. In contrast, geldanamycin reduced the interaction of Hsp90 with Akt by only 25% relative to the amount of His-tagged [^{35}S]-Akt immunoadsorbed from the reticulocyte lysate.

We next examined the effect of geldanamycin on the interaction of Hsp90 and Cdc37 with Akt in differentiating C2C12 cells. Western blot analysis of protein that co-adsorbed with Akt from C2C12 extract indicated that the interaction of Hsp90 with Akt was not disrupted by geldanamycin. However, the interaction of Cdc37 with Akt was decreased ~50 % relative to the DMSO-treated control cells (Fig. 13C).

Effect of short-term geldanamycin treatments on Akt phosphorylation-state in proliferating and differentiating C2C12 cells. The results describe above indicate that short-term treatments of C2C12 cells with geldanamycin have little effect on the turnover rate of mature Akt molecules and does not disrupt the interaction of Hsp90 with Akt over the period of drug treatment. Therefore, we examined the effect of geldanamycin treatment on the phosphorylation state of Akt in proliferating and differentiating C2C12 cells. Phosphorylation of Akt on Thr^{308} and Ser ^{473} is associated with its activation, which occurs through a number of pathways as described in the introduction (153, 159).

Short-term (30 min) treatment of proliferating C2C12 with geldanamycin caused no loss of Akt protein, and did not affect the level of Akt phosphorylation on Thr^{308} (Fig.

14A). However, an approximate doubling of Akt phosphoryation on Ser^{473} was noted in the presence of geldanamycin, which correlated with an increase in the level of phosphorylation of glycogen synthase kinase-3β (GSK3) (Fig. 14A), a well characterized target of Akt (153). Thus, short-term treatment of actively growing C2C12 cells with geldanamycin appeared to be causing the activation of Akt.

Subsequently, we compared the effect of geldanamycin on Akt activity and phosphorylation state in proliferating versus differentiating C2C12 cells. Again, geldanamycin-treatment of growing C2C12 cells (GC) caused no decrease in Akt protein, caused a slight increase in the phosphorylation of Thr^{308}, and clearly increased the level of Akt phosphorylation on Ser^{473} (Fig. 14B). On the higher resolution gel used in this experiment, the increase in Akt phosphorylation on Ser^{473} correlated with the appearance of a distinct doublet on the western blot used for the detection of total Akt protein.

Short-term treatment of differentiated C2C12 cells (DF) with geldanamycin also did not cause any decrease in Akt protein levels and increased the level of Akt phosphorylation on Ser^{473} (Fig. 14B). However, phosphorylation of Akt on Thr^{308} was not observed in either control or geldanamycin-treated differentiating C2C12 cells. Thus, Akt activity in differentiated C2C12 cells appears to be regulated at the level of Ser^{473} phosphorylation and not through changes in the phosphorylation state of Thr^{308}.

Effect of Hsp90 inhibition on Akt phosphorylation-state in differentiating C2C12 cells. To determine whether C2C12 activation was a specific effect of geldanamycin, or was a general effect of the inhibition of Hsp90, we compared the effects of geldanamycin to those of novobiocin, an Hsp90 inhibitor which binds to the C-terminus of Hsp90 (26-28).

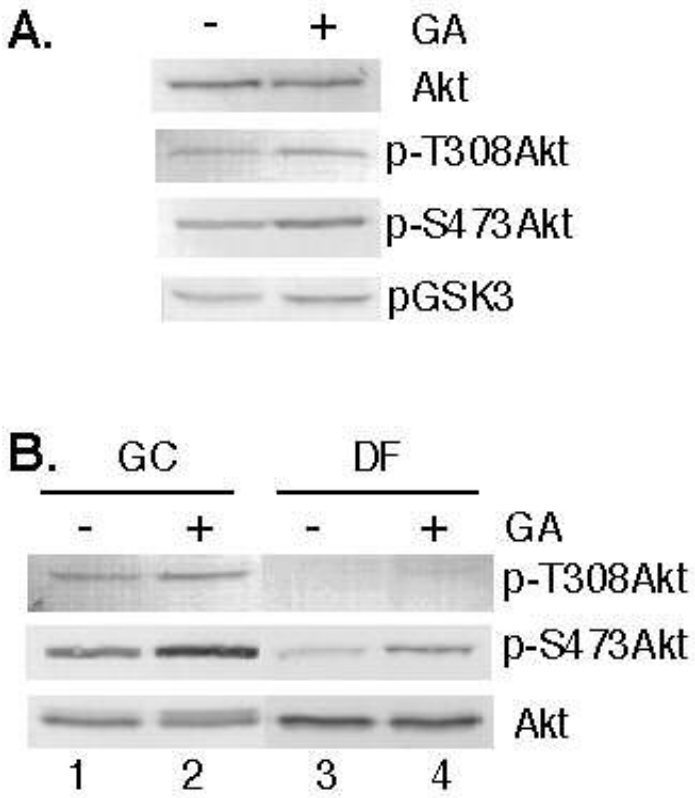

Figure 14. Differential phosphorylation states of Akt in proliferating and differentiating C2C12 cells. (A) Cells were grown to 50 to 70% confluence and treated with DMSO (-) or 5µg/mL geldanamycin (GA, +) for 30 min. (B) C2C12 were grown to 50 to 70% confluence (growing cells, GC: lanes 1 and 2), or induced to differentiate (differentiating cells, DF: lanes 3 and 4) as described in Figure 12. Cells were treated with DMSO (-, lanes 1 and 3) or 5 µg/mL geldanamycin (GA, +, lanes 2 and 4). Cell lysates were prepared as described under "Experimental Procedures", and samples were analyzed by western blotting with anti-Akt (Akt), anti-Akt/phospho-Thr^{308} (p-T308Akt), anti-Akt/phospho-Ser^{473} (p-S473Akt) Akt, or anti-GSK3 phospho-S$^{21/9}$ (p-GSK3) specific antibodies.

Again, treatment of C2C12 cells with geldanamycin led to enhanced phosphorylation of Akt on Ser ^{473}, which increased as early as 30 min after initiation of the drug treatment compared to the DMSO treated control cells (Fig. 15A). Novobiocin similarly enhanced the phosphorylation of Akt on Ser ^{473} (Fig. 15B), and the enhancement of Ser ^{473} phosphorylation in C2C12 cells occurred in a concentration dependent manner. Thus, inhibition of Hsp90 function with either geldanamycin or novobiocin treatment led to enhancement of Akt phosphorylation. This enhanced phosphorylation of Akt correlated with increased Akt activity, as evidenced by enhanced phosphorylation state of GSK3β in C2C12 cells (Fig. 14A and 15B).

Previous studies have indicated that activation of Akt by phosphorylation on Ser ^{473} may occur in the absence of PDK1 activation (99, 153). PP2Ac has been proposed to be the most likely candidate for regulating the rate of Akt dephosphorylation (99, 137, 175, 177). To test this hypothesis, differentiating C2C12 cells were treated with okadaic acid or geldanamycin. Okadaic acid treatment enhanced Akt phosphorylation on Ser ^{473} in a concentration dependent manner, similar to the effect observed for geldanamycin (Fig. 15C). Again, enhanced Akt phosphorylation on Ser^{473} correlated with enhanced GSK3β phosphorylation (Fig. 15C). These results suggest that Akt activation in response to geldanamycin and novobiocin may be occurring through their capacity to inhibit the ability of PP2Ac to dephosphorylate Akt via some as yet uncharacterized mechanism.

Interaction of PP2Ac with Akt in C2C12 cells. While PP2A has been proposed to play a role in regulating the activity of Akt, only one previous study has demonstrated a direct interaction between the two proteins (179). To further characterize the effect of

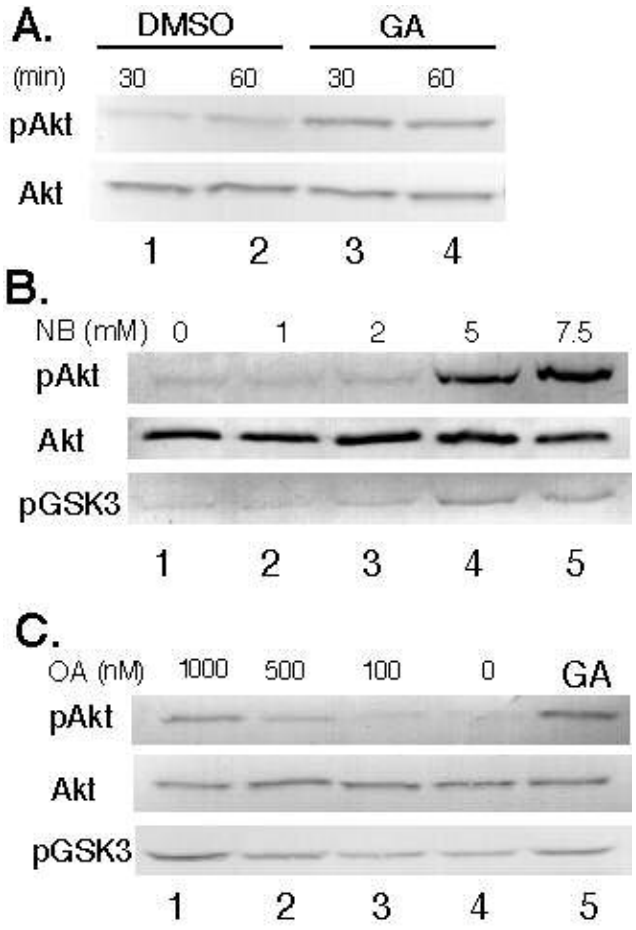

Figure 15. Effects of short-term treatments with geldanamycin, novobiocin, and okadaic acid on Akt phosphorylation and activity. Cells were maintained in DM for 40 hrs and then treated with DMSO (lane 1 & 2) or 5µg/mL geldanamycin (GA, lane 3 & 4) for 30 or 60 min (A); or cells were treated with DMSO (lane 1) or 1 mM (lane 2), 2 mM (lane 3), 5 mM (lane 4) or 7.5 mM (lane 5) novobiocin (NB) for 30 min (B); or cells were treated with DMSO (lane 4), or 5 µg/mL:9 µM geldanamycin (GA, lane 5), or 1000 nM (lane 1), 500 nM (lane 2), or 100 nM (lane 3) okadaic acid (OA) for 30 min (C). Cells were lysed with SDS buffer and analyzed by western blotting with anti-Akt (Akt), anti-Akt/phopho-Ser^{473} (pAkt) Akt, or anti-GSK3 phospho-S$^{21/9}$ (pGSK3) specific antibodies.

geldanamycin on Akt activation, we examined whether Akt and PP2Ac were associated in C2C12 extracts. PP2Ac was found to be co-immunoadsorbed with Akt from C2C12 extracts (Fig. 16A). Geldanamycin-treatment had no effect on the interaction of the two proteins and did not have an effect on the amount of Hsp90 that was co-adsorbed with Akt from the cell extracts. Furthermore, Akt co-immunoadsorbed with PP2Ac in the reciprocal experiment (Fig. 16B). Thus, PP2Ac interacts directly with Akt in C2C12 cells and inhibition of Hsp90 function with geldanamycin had no effect on this interaction.

Discussion

Our results indicate that inhibition of Hsp90 function by geldanamycin results in the progressive loss of Akt protein from differentiating C2C12 cells. However, the effect of geldanamycin was only reproducibly observed after 8 h of drug treatment. While our results are consistent with previously published studies noting a loss in Akt protein levels after 9 h of treatment with 17-AAG (48, 184), the previously published pulse-chase study only examine mature Akt levels after treatment of cells with 17-AAG for 12 h or longer (48). Since we observed little effect of geldanamycin on the interaction of Hsp90 with Akt, and geldanamycin-treatment results in the inhibition of a myriad of Hsp90-dependent proteins, it is difficult to conclude that the geldanamycin-induced decrease in Akt levels is a direct effect of geldanamycin on Akt stability via Hsp90-inhibition.

On the contrary, our data indicate that the geldanamycin-induced decrease in Akt levels may be primarily the result of inhibition of Hsp90-facilitated maturation of Akt.

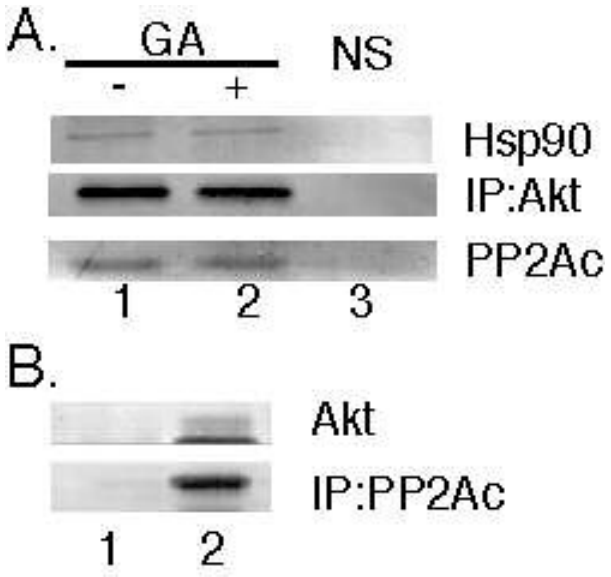

Figure 16. Interaction of Akt with PP2Ac. Confluent C2C12 cells were maintained in DM for 40 h (A and B), and were untreated (B) or were treated with DMSO (A: lane 1) or 5 µg/mL geldanamycin (A: GA, lane 2) for 30 min. Cell lysates were prepared as described under "Experimental Procedures" and (A) Akt was immunoadsorbed with anti-Akt agarose resin (IP:Akt) or (B) PP2A was immunoadsorbed with anti-PP2Ac antibody (IP:PP2Ac). Samples were analyzed by western blotting with anti-PP2Ac, anti-Akt, or anti-Hsp90 antibodies. Nonspecific IgG agarose resin used as a control for nonspecific binding of proteins to the immune resin (A, NS: lane 3; and B, lane 1).

Geldanamycin-treatment nearly quantitatively blocked the maturation of newly synthesized Akt in differentiating in C2C12 cells. Furthermore, newly synthesized immature Akt molecules were completely degraded within 1 h of their synthesis in geldanamycin treated cells. Thus, the slow progressive loss of Akt in the presence of geldanamycin correlates with the inability of the cells to mature newly synthesized Akt molecules into a stable conformation. However, as discussed below, the geldanamycin-induced enhancement of the turnover of mature Akt molecules may result from its ability to cause the activation of Akt.

The geldanamycin-induced inhibition of Akt maturation probably results from its ability to inhibit Cdc37 from productively interacting with newly synthesized Hsp90-bound Akt moleules in cultured C2C12 cells, rather than geldanamycin affecting the interaction of Hsp90 and Cdc37 with mature Akt molecules. Both Hsp90 and Cdc37 are required to facilitate the maturation and activation of numerous protein kinases in cells (35), and consistent with the results reported here, both Hsp90 (48, 99) and Cdc37 (48, 193) interact with endogenous Akt in cells. Previously, we have shown that while geldanamycin treatment does not block the interaction of Hsp90 with protein kinases, it does, however, cause Hsp90-kinase binding to become labile to washing in high salt buffers (28, 41, 45, 129, 141, 194). On the other hand, geldanamycin blocks the interaction of Cdc37 with their newly synthesized target kinases (28, 41, 45, 129, 141, 194). In our current studies, short-term treatment with geldanamycin had little effect on the interaction of Hsp90 with Akt in differentiating C2C12 cells, while geldanamycin reduced the interaction of Cdc37 with Akt by ~50%. In a previous study, treatment of MCF-7 cells with 17-AAG had little effect on the interaction of Cdc37 with Akt, but data relative to the effect of 17-AAG on Hsp90-Akt interactions was not shown(48) . In the

reticulocyte lysate model system, geldanamycin blocked the interaction of Cdc37 with newly synthesized Akt, while it had much less of an effect on the interaction of Hsp90 with Akt. Thus, geldanamycin-induced inhibition of Akt maturation is likely the result of its ability to inhibit the binding of Cdc37 to complexes formed between Hsp90 and newly synthesized Akt molecules.

Our data suggest that turnover of Akt occurs via the proteasome. Treatment of C2C12 cells with lactacystin, a proteasome-specific inhibitor, and ALLN, a more generalized protease inhibitor, prevented the complete loss of mature Akt molecules from differentiating C2C12 cells treated with geldanamycin. The partial loss of Akt observed in the presence of the inhibitors is likely due to accumulation of ubiquitinated Akt, which would not have been detected under our western blot conditions. Again, these results are consistent with conclusions drawn from another study examining the Hsp90-dependence of Akt stability in tumor cell lines (48), which clearly indicated that inhibition of Hsp90 by ansamycins leads to a loss of Akt protein from cells via the ubiquitination/proteasome pathway. While alterations of PP2A activity (99) or PDK1 levels (185) could undoubtedly play additional roles in the decline of cellular Akt activity observed upon Hsp90-inhibition, as proposed by Tsuruo and co-workers, the loss of total Akt activity observed upon long-term (24 h) inhibition of Hsp90 function is most simply accounted for by the loss of mature Akt protein.

Akt activity appears to be regulated differently in proliferating versus differentiating C2C12 cells. Akt was phosphorylated on Thr^{308} and Ser^{473} in pre-confluent C2C12 cells, but was only phosphorylated on Ser^{473} in differentiating cells. Phosphorylation of Akt at Thr^{308} has been shown to be stimulated through growth factor

receptor (e.g., insulin-like growth factor-1 receptor) mediated pathways [reviewed in (153, 155-157, 159)]. Phosphorylation of Thr^{308} is then thought to enhance phosphorylation of Akt at Ser^{473}. Our results appear to indicate that differentiating C2C12 cells are able to maintain the ability to regulate Akt activity via changes in Ser^{473} phosphorylation after withdrawal of growth factors from the serum and in the absence of phosphorylation of Thr^{308}. These results are consistent with previous studies indicating that after its initial activation, phosphorylation of Thr^{308} is not required to maintain the activity of Akt (153, 170).

Our results indicate that Akt activity is modulated both by positive and negative signaling pathways in differentiating C2C12 myoblasts. Akt function is required for muscle cell differentiation, and in maintaining the viability and size of differentiated muscle cells [reviewed in (154-159)]. As noted in the introduction, Akt activity is regulated through multiple mechanisms, and can involve mechanisms leading to stimulation of Akt's phosphophorylation or dephosphorylation (153, 159, 169, 176, 179, 189). Stimulation of Akt leads to skeletal and cardiac muscle hypertrophy, while down-regulation of its activity leads to muscle cell atrophy and apoptosis (160-163). Treatment of differentiating C2C12 cells with okadaic acid leads to enhanced phosphorylation of Akt on Ser^{473} and increased GSK3β phosphorylation. Thus, positive signals that lead to Akt phosphorylation on Ser^{473} in C2C12 cells are being balanced by negative signals, which stimulate Akt dephosphorylation. While this manuscript was in preparation, geldanamycin was also reported to enhance Akt phosphorylation in IGF-1 (or insulin) stimulated neuroblastoma and embryonic kidney cells, possibly through a mechanism involving inhibition of Akt dephosphorylation (178). Our results are consistent with this

hypothesis, and identify PP2Ac as the phosphatase responsible for Akt dephosphorylation modulated by geldanamycin- and novobiocin-inhibitable Hsp90 function in C2C12 cells.

Hsp90 appears to play a role in balancing the phosphorylation state of mature Akt molecules in response to signaling pathways. As opposed to the long-term effects of Hsp90 inhibitors on Akt stability and protein levels, short-term treatment of differentiating C2C12 cells with geldanamycin or novobiocin led to enhanced Akt activity, as measured by Akt phosphorylation at Ser^{473} and enhanced phosphorylation of GSK3β. The effect of the Hsp90 inhibitors on Akt phosphorylation appears to be through their action on Hsp90, as the inhibitors had no direct inhibitory effect on PP2Ac activity in vitro (not shown). Furthermore, PP2A appears to interact directly with Akt, rather than Hsp90, since no detectable amounts of Hsp90 or PP2Ac were co-immunoadsorbed with anti-PP2Ac or anti-Hsp90 antibodies, respectively. Thus, inhibition of Hsp90 function in C2C12 cells appears to alter Akt structure in a manner that interferes with the ability of PP2A to dephosphorylate Akt at Ser^{473}, as geldanamycin does not interfere with the interaction of Akt with PP2A or Hsp90. Alternatively, geldanamycin could cause the inhibition of an Hsp90-dependent protein whose activity is required for PP2Ac activity.

Geldanamycin-induced activation of Akt may also account for the reduced half-life of mature Akt molecules in differentiating C2C12 cells. Activated mutants of Src-family kinases, v-Src, Lck/Y505F and Hck/Y499F have an increased dependence upon Hsp90 function to maintain their stability and function in their mature state, and geldanamycin enhances the rate of turnover of these proteins in cells relative to their wild type counterparts. Thus, mutations that lead to hyperactivation of Hsp90-dependent kinases appear to generate a greater dependence of these kinases on Hsp90 to maintain

their function. The enhanced turnover rate of mature Akt in geldanamycin treated cells may similarly be a function of its enhanced state of activation.

As alluded to above, the results presented here also suggest an additional mechanism by which inhibition of Hsp90 function can enhance cell survival and protect against apoptosis in certain cell populations. Depending on cell context, geldanamycin can promote or impede apoptosis [reviewed in (195)]. Treatment of both rat neonatal cardiomyocytes and myogenic H9c2 cells with Hsp90 inhibitors, herbimycin A or geldanamycin induced resistance of these cells to apoptosis stimulated by ischemia (196). Geldanamycin also protects brain cells from damage induced by ischemia (189, 197). While the anti-apoptotic effect of Hsp90 inhibitors has been attributed primarily to their ability to induce the elevation of Hsp70 levels, our results suggest an additional mechanism may also play a role, at least during short-term treatment with these drugs: the potential anti-apoptotic effect of Akt stimulation induced by short-term inhibition of Hsp90.

The hypothesis that Hsp90 functions to balance the phosphorylation state, and therefore, attenuate the activation of the Akt kinase may be relevant to the regulation of other protein kinase systems. After their Hsp90-dependent maturation, geldanamycin has been shown to activate the double-stranded RNA-activated eIF2α kinase, and the Raf-MAPK pathway in cells (198). The maturation of the HRI kinase activity has been shown to involve a balance between the stimulatory effects of Hsp90-Cdc37 on HRI's autophosphorylation, and the inhibitory effects of HRI's Hsp90- and PP5-dependent dephosphorylation (41, 129). Thus, the capacity of Hsp90 to modulate a protein's

activation state by integrating the signals from both positive and negative effectors may be paradigm applicable to other Hsp90-dependent signal transduction proteins.

CHAPTER IV

Differential effects of Hsp90 inhibition on protein kinases regulating signal transduction pathways required for myoblast differentiation

Abstract

Several protein kinases whose functions are required for the development and homoeostasis of muscle cells are dependent upon Hsp90 to acquire and maintain an active conformation. These protein kinases not only regulate pathways involved in myoblast differentiation, they also regulate growth and viability of differentiated myotubules. In this study, we examined the effect of geldanamycin-induced inhibition of Hsp90 function on the differentiation of C2C12 myogenic cells. Geldanamycin treatment of differentiating C2C12 myoblasts, blocked myotubule formation, and led to the depletion of three Hsp90-dependent protein kinases, ErbB2, Fyn, and Akt, and induction of apoptosis. Geldanamycin caused ErbB2 levels to drop rapidly (absent within 8 h), while Fyn and Akt levels decreased at a slower rate (absent after 24 h). Geldanamycin blocked the interaction of Hsp90 and Cdc37 with Fyn, an interaction that has not been previously documented. Pulse-chase experiments with metabolically [^{35}S]-labeled proteins indicated that newly synthesized Akt and Fyn were degraded rapidly in the presence of geldanamycin. However, geldanamycin had little effect on the turnover rate of mature Fyn and Akt. Curiously, total cellular Src (c-Src) protein levels and the turnover rate of newly synthesized [^{35}S]-c-Src were not affected by geldanamycin.

Geldanamycin blocked the expression of myogenin in differentiating C2C12 cells without affecting the level of MyoD expression. However, MyoD was found to interact with Cdc37, and geldanamycin disrupted this interaction. Thus, inhibition of Hsp90 disrupts signal transduction networks that are involved in regulating myoblast differentiation, and muscle cell homeostasis. Our findings indicate that protein kinases can have a differential dependence on Hsp90 for maintenance of their function, and that Hsp90-inhibitors could potentially have both beneficial and adverse effects on muscle cell populations depending upon the physiology of the system.

Introduction

Muscle cell differentiation is a highly complex and tightly regulated process that requires multiple steps to occur in an ordered progression [reviewed in (199-204)]. Myoblasts must withdraw from the cell cycle, elongate and then fuse into multinucleated myotubes. This program of differentiation requires the function of a number of signal transduction cascades that regulate the activation of muscle-specific regulatory factors, and the expression of muscle specific genes, which promote the development of mature muscle tissue. In addition, signal transduction pathways subsequently function to regulate the size and sustain the viability of the mature muscle cell (reviewed in (153, 155-159)).

The proto-oncogene product ErbB2 (a.k.a., HER2) encodes a receptor tyrosine kinase that acts as a co-receptor for growth factors through hetero-dimerization with other members of the erbB-family [reviewed in (205, 206)]. Signaling pathways regulated by ErbB2 play critical roles in numerous developmental processes. ErbB2 function is

required for the proper development and differentiation of cardiac and skeletal muscle. Furthermore, ErbB2 function is also required for cardiac and skeletal muscle cell homoeostasis, and for the survival of myoblasts (207). Disruption of ErbB2 function leads to apoptosis of differentiated myofibrils, with ensuing defects in skeletal muscle regeneration due to a reduction in the number of muscle spindles (208). ErbB2 is also required to maintain normal heart physiology, and defects in ErbB2 function result in cardiomyopathy and heart failure (205, 206, 208, 209).

While insulin-like growth factors (e.g., IGF-1) act in concert with other growth factors to promote myoblast proliferation, they are also potent stimulators of muscle cell differentiation (160, 164, 167, 210, 211). IGFs act in part through stimulation of the PI3K/Akt signal transduction pathway (153, 161, 167, 168, 210, 212). Akt1 activation is required for the expression of Akt2, and subsequent Akt2-dependent activation of MyoD-MEF2 transcriptional activity and induction of myogenin expression (161, 165, 166, 168, 186, 210). Activation of Akt also promotes muscle cell growth and increase in muscle mass, while Akt deficiency leads to atrophy of muscle tissue (155, 157-163, 172, 187, 212-217).

Cell adhesion also plays a part in the highly orchestrated multi-step process of muscle cell differentiation (reviewed in (153, 218)). Integrins are hetero-dimeric transmembrane receptors composed of alpha- and beta subunits that interact with components of the extracellular matrix and regulate cell adhesion. Different isoforms of these subunits are expressed in a developmentally regulated fashion, are localized to different sites in cells, and carry out distinct functions (183, 219-225). Signaling via integrin receptors modulates cell cycle progression, migration and proliferation of developing myoblasts. Integrin receptors are also involved in regulating the

differentiation of myoblasts. Integrins play roles in myotubule formation via cell fusion, the formation and the maintenance of the integrity of neuromuscular and myotendinous junctions, and the adhesion of muscle fibers that is essential for generation of contractile force (218, 222, 224-227). Signaling pathways activated by integrin receptors both promote and impede the differentiation of myoblasts. The PI3K/Akt pathway is both activated and inhibited by this receptor family via changes in the activities of protein kinases and phosphatases that promote the phosphorylation or dephosphorylation of Akt (169, 176, 179, 182, 211, 221, 222, 228-230). Dysregulation of intergrin-mediated signaling is associated with both skeletal and cardiac muscle hypertrophy and atrophy (219, 220, 223).

Recent studies have indicated that non-receptor Src-family kinases are also involved in the regulation of muscle cell proliferation, differentiation and survival (231-233). c-Src stimulates myoblast proliferation, and also appears to play a role in clustering of acetylcholine receptors during development of the neuromuscular junctions (234-237). Expression of the Src-family kinase Fyn is up-regulated during myoblast differentiation and myotube formation (234, 238, 239). Fyn expression is required for cell fusion leading to the formation of multi-nucleated muscle cells and neuromuscular junctions. Promotion of myofiber survival and suppression of anoikis (apoptosis induced by disruption of cells adhesion to the extracellular matrix) also involves Fyn signaling modulated through the merosin-integrin cell adhesion system (238).

The integration of signals generated through the pathways described above leads to changes in the expression or activity of gene products that regulated the expression of muscle-specific genes and cell differentiation. Skeletal muscle differentiation can be induced by the overexpression of myogenic factors, such as MyoD, myogenin, Myf5 and

MRF4 in non-muscle cells [reviewed in (200-203, 240)]. Inhibition of the expression or function of these myogenic factors can arrest the formation of multi-nucleated cells during differentiation of C2C12 myoblasts, indicating that these factors regulate the expression of a distinct subset of muscle-specific genes at the onset of cell differentiation (166, 182, 186, 210, 234).

Hsp90 (heat shock protein 90) is an ATP dependent molecular chaperone, which facilitates the folding and activation of numerous proteins involved in signal transduction in cells [reviewed in (13, 29, 101)]. Hsp90 signal transduction clients carry out functions that are involved regulation of cell proliferation, differentiation, and survival. Included in this myriad of Hsp90-dependent protein clients are [reviewed in (13, 29, 101)] transcription factors, such as steroid hormone receptors, hypoxia-inducible factor-alpha, and MyoD (241, 242); protein kinases, including ErbB2, Akt, and Src family kinases; and others proteins, such as nitric oxide synthase and telomerase.

Drugs that bind to the N-terminal ATP-binding domain of Hsp90, such as the ansamycin-derivative geldanamycin, and radicicol, inhibit Hsp90 function (reviewed in (13, 29, 101, 110, 180). Inhibition of Hsp90 can prevent the maturation of Hsp90-dependent clients, and induce the destabilization and degradation of Hsp90 clients. Tumor cell populations are particularly sensitive to apoptotic cell death induced by these Hsp90-inhibitors (100, 243-246).

Most studies on the physiological effects of Hsp90-inhibition have been carried out on cancer cell populations. The geldanamicin derivative 17-allylamino-geldanamycin is currently in phase-I and -II clinical trials (110, 180, 243, 244, 246). Considering the fact that inhibition of Hsp90 function interferes with numerous signal transduction

pathways that regulate the proliferation, differentiation and survival of other cell populations, a greater understanding of the potential adverse effects of Hsp90-inhibition on normal cells, tissues and organs is needed. In this report, we have examined the effect of inhibition of Hsp90 on myoblast differentiation. Our results indicate that Hsp90 function is essential for the cellular processes involved in myoblast differentiation, as it is required for the expression of the muscle regulatory factor (e.g. myogenin), and for the maturation and/ or stability of the ErbB2, Akt, and Fyn kinases.

Experimental Procedures

Cell culture – C2C12 myoblast (from ATCC) cells were grown in DMEM (BioWhitaker) containing 10% fetal calf serum (Hyclone) and streptomycin/penicillin (100 units/mL, Sigma) at 37ºC, in the presence of 5% CO_2 and 95% air. For induction of differentiation, confluent cells were maintained in DMEM containing 5% equine serum (Hyclone) (differentiation media, DM). Cells were photographed (100X magnification) at 50-70% confluence, at confluence, 40 h after induction of differentiation, and 24 h after treatment of the 40 h differentiating cells with DMSO or 5 μg/mL geldanamycin.

Analysis of the effects of geldanamycin on differentiating C2C12 myoblasts – Confluent cells were maintained in DM for 40 h, and then treated with the given pharmacological agent or solvent control for 0 to 24 h as indicated in the legend to the figures. After washing the cells briefly with phosphate-buffered saline (PBS), the cells were lysed with SDS sample buffer. Samples were boiled in a water bath for 5 min, separated by SDS-PAGE, and electro-transferred to PVDF membrane (Bio-Rad). Protein concentration for SDS-PAGE was determined with a BCA assay kit (Pierce). Cell

protein levels were analyzed by western blotting with anti-ErbB2 (Oncogene Research), anti-Hsp90, anti-Akt, anti-cleaved poly (ADP-ribose) polymerase (PARP) (Asp214) (Cell Signaling), anti-Fyn and c-Src (Santa Cruz Biotechnology) antibodies.

Pulse-chase analysis of Fyn, Akt and c-Src expression – Confluent cells were maintained in DM for 40 h, followed by metabolic labeling with [^{35}S]methionine (100 µCi/mL, PerkinElmer). For experiments analyzing the turnover of newly synthesized kinases, geldanamycin (5 µg/mL) or vehicle control (DMSO) was added to the DM during the 30 min labeling period with [^{35}S]methionine. After 30 min, the cells were washed and maintained in fresh DM containing geldanamycin (5 µg/mL) for 0 to 4 h. For experiments analyzing the turnover of mature kinases, cells were incubated with [^{35}S]methionine for 30 min. The cells were then washed and maintained in fresh DM medium for 3h. Geldanamycin (5 µg/mL) or DMSO was then added for 0 to 24 h. At the time points indicated in the figure legends, cells were washed with phosphate-buffered saline (PBS), and then lysed with RIPA lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 0.25% deoxycholic acid, 1% SDS, 2mM EDTA, 1mM EGTA and protease inhibitors cocktail (Sigma)). Samples were clarified by centrifugation in a microcentrifuge for 15 min at maximum speed (~12000 rpm), and each kinase was immunoadsorbed with anti-Akt, anti-Fyn, or anti-c-Src antibodies at 4ºC for 2~3 h. Samples were washed 3 times with lysis buffer, boiled in SDS sample buffer and analyzed by SDS-PAGE on 8% gels. Proteins were electro-transferred to PVDF membrane, and total level of kinase in each sample was determined by western blotting

with anti-Akt, anti- Fyn, and anti-c- Src antibodies. [^{35}S]-labeled Akt, Fyn, and c-Src were visualized by autoradiography.

Co-immunoadsorption of Fyn or MyoD with Hsp90/Cdc37 complex– Confluent cells were maintained in DM for 40 h as described above. Cells were treated with geldanamycin (5 µg/mL) or DMSO for 3 h and then lysed in buffer containing 50 mM HEPES pH 7.5, 100 mM NaCl, 0.1% deoxycholic acid, 0.1% Triton X-100, 20 mM sodium molybdate, 50 mM NaF, 2 mM EDTA, 1 mM EGTA, and protease inhibitor cocktail (Sigma). Cell lysate was clarified by centrifugation for 20 min at ~ 12000 rpm. Fyn or MyoD was immunoadsorbed with anti-Fyn or anti-MyoD antibodies at 4º C for 2~3h. Samples were washed 4 times with washing buffer (10 mM PIPES pH 7.2, 150 mM NaCl) and then analyzed by western blotting with anti-Hsp90, anti-Cdc37, anti-Fyn or anti-MyoD antibodies as indicated in the figure legends.

Analysis of the effects of geldanamycin on the expression of myogenic factors in differentiating C2C12 cells – Confluent cells were maintained in DM and treated with 5 µg/mL geldanamycin for 20 h at 0, 15, 24 h after induction of differentiation. After briefly washed with phosphate-buffered saline (PBS), cells were lysed in SDS sample buffer and then analyzed by SDS-PAGE on 8% gels. Protein concentration for SDS-PAGE was determined with a BCA assay kit. The levels of proteins were determined by western blotting with anti- myogenin and anti-Akt1 (Santa Cruz biotechnology), anti-Akt2 (Upstate Biotechnology), and anti-MyoD antibodies.

Results

The effects of Hsp90 inhibition on signal transduction molecules in differentiating C2C12 cells - Upon reaching confluence, C2C12 cells undergo differentiation in response to lowering serum growth factors by the replacement of fetal calf serum in the growth medium with horse serum (181, 183, 199, 225, 247). Upon changing medium, cells withdraw from the cell cycle and either differentiate and fuse into multinucleated myotubules or become quiescent "reserve cells" (199, 223, 248) that share several characteristics of muscle pioneer cells. As noted in the introduction, two known Hsp90-dependent client molecules, ErbB2 (173, 184, 188, 249, 250) and Akt (48, 99, 184, 185), play critical roles in the regulation of signaling cascades that control muscle cell proliferation, survival, and differentiation.

Since inhibition of Hsp90 function has been demonstrated to block the function of Hsp90-dependent clients, either through inhibition of the client's maturation or through their destabilization and subsequent depletion from cells (13, 101, 110, 180), we examined the effect of geldanamycin-induced inhibition of Hsp90 function on signal transduction molecules that are involved in myoblast differentiation and the development of skeletal muscle: ErbB2, Akt, c-Src and Fyn.

Differentiation of confluent C2C12 cells was induced for 48 h, followed by treatment of the cells with geldanamycin or its vehicle solvent DMSO. At the times indicated, cell extracts were prepared for analysis of ErbB2, Akt, c-Src, and Fyn levels by western blotting. Consistent with previous studies in proliferating cancer cells (188, 189, 249), the amount of ErbB2 present in differentiating C2C12 was decreased rapidly, with ErbB2 becoming undetectable after 8 h of treatment of cells with geldanamycin (Fig 17).

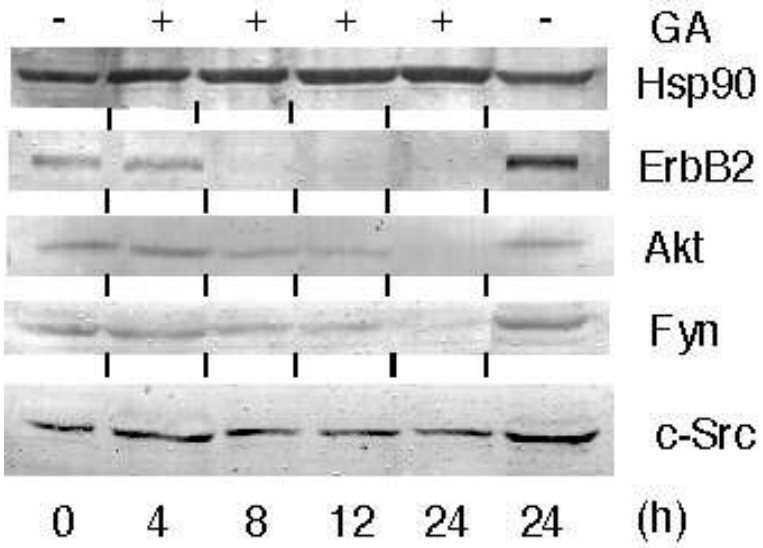

Figure 17. Effects of geldanamycin on signal transduction molecules in differentiating C2C12 myoblasts. Confluent cells maintained in DM were treated with DMSO or 5 µg/mL geldanamycin for the time indicated in the figure (0 to 24 h). Cells were lysed directly with SDS sample buffer. The level of each protein was determined by western blotting with anti- Hsp90, anti-ErbB2, anti-Akt, anti- Fyn, and anti-c-Src antibodies.

In contrast, the amounts of Akt and Fyn protein declined gradually, becoming undetectable after 12 to 24 h of treatment with geldanamycin. Interestingly, the level of c-Src was maintained in cells even after 24 h of geldanamycin treatment. Thus, over a period of 24 h, inhibition of Hsp90 function lead to the depletion of multiple cellular protein kinases that are known to play critical roles in regulating muscle cell differentiation and survival.

Microscopic examination of control C2C12 cells indicated that they had an elongated morphology 48 h after culturing the cells in media containing horse serum, indicating that the cells were in the first stages of differentiation (Fig 18C). After an additional 24 h treatment with DMSO, wide and elongated multi-nucleated cells were observed, indicative of the initial stages of myotubule formation (Fig. 18D). In contrast, 24h after treatment of C2C12 cells with geldanamycin, the majority of the cells remained thin and spindle-like (Fig. 18E). The multi-nucleated cells that were present were shorter and thinner than those grown under control conditions. In addition, areas rich in rounded cells populations,suggestive of cells that were undergoing apoptosis, were readily apparent. Western blotting of cell extracts prepared from the control and geldanamycin treated cells with anti-cleaved PARP antibody, a marker for the initial stage of programmed cell death, confirmed that the geldanamycin-treated cells were undergoing apoptosis (Fig. 18F).

Inhibition of Hsp90 function destabilizes newly synthesized Fyn and Akt - Previous studies have documented that inhibition of Hsp90 function causes the accelerated turnover of both newly synthesized and mature ErbB2 via proteasome-

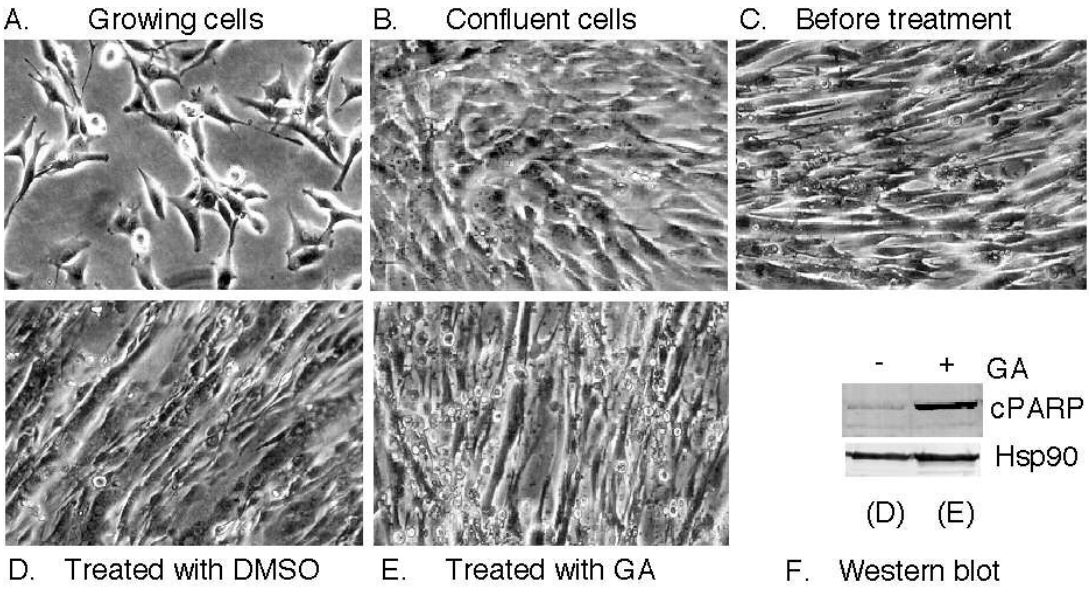

Figure 18. Effects of geldanamycin on differentiation of C2C12 myoblasts. C2C12 cells were photographed: at 50-70% confluence (A: Growing cells); at confluence (B: Confluent cells); 2 days after induction of differentiation (C: Before treatment); and after 24 h of treatment of C2C12 cells, previously induced to differentiate for 2 days, with DMSO (D) or or 5 µg/mL geldanamycin (E: Treated with GA). (F) western blot (WB) analysis of extracts prepared from the cells shown in panel D and E of the figure with anti-cleaved PARP antibody.

mediated degradation (87, 188, 189, 249, 251, 252). The rapid depletion of ErbB2 from cells is a consequence of the dependence of mature ErbB2 on Hsp90-function. The data presented above suggest that the Src-family kinase Fyn is likely an Hsp90-dependent kinase, but c-Src appeared to be Hsp90-independent, as geldanamycin treatment did not reduced c-Src levels in differentiating C2C12 cells.

Pulse-chase experiments were therefore carried out to determine the Hsp90-dependence of the maturation and stability of newly synthesized Fyn, c-Src and Akt kinases. C2C12 cells were pulse-labeled with [^{35}S]-Met in the presence or absence of geldanamycin for 30 min, followed by incubation in fresh media with or without the addition of geldanamycin for 0 to 4 h. Fyn, Akt and c-Src were immunoadsorbed from cell extracts prepared at the indicated times after the initiation of the chase, and samples were then analyzed by SDS-PAGE and autoradiography to determine the effects of Hsp90 inhibition on the turnover rate of each kinase.

While newly synthesized [^{35}S]-Fyn was stabile in control cells, newly synthesized [^{35}S]-Fyn was nearly completely degraded within 3 h when matured in the presence of geldanamycin (Fig. 19A). Similarly, newly synthesized [^{35}S]-Akt was nearly completely degraded in cells within 1 h of chase in the presence of geldanamycin (Fig. 19C). In contrast geldanamycin appeared to have no effect on the turnover of newly synthesized c-Src in differentiating C2C12 cells (Fig. 19E). These observations indicate that Hsp90 function is required for folding and stability of newly synthesized Fyn and Akt molecules in differentiating C2C12 myoblasts. Consistent with the absence of an effect of geldanamycin on the total cellular levels of c-Src observed in Figure 17, the turnover rate of newly synthesized c-Src did not appear to be affected by treatment of the cells with

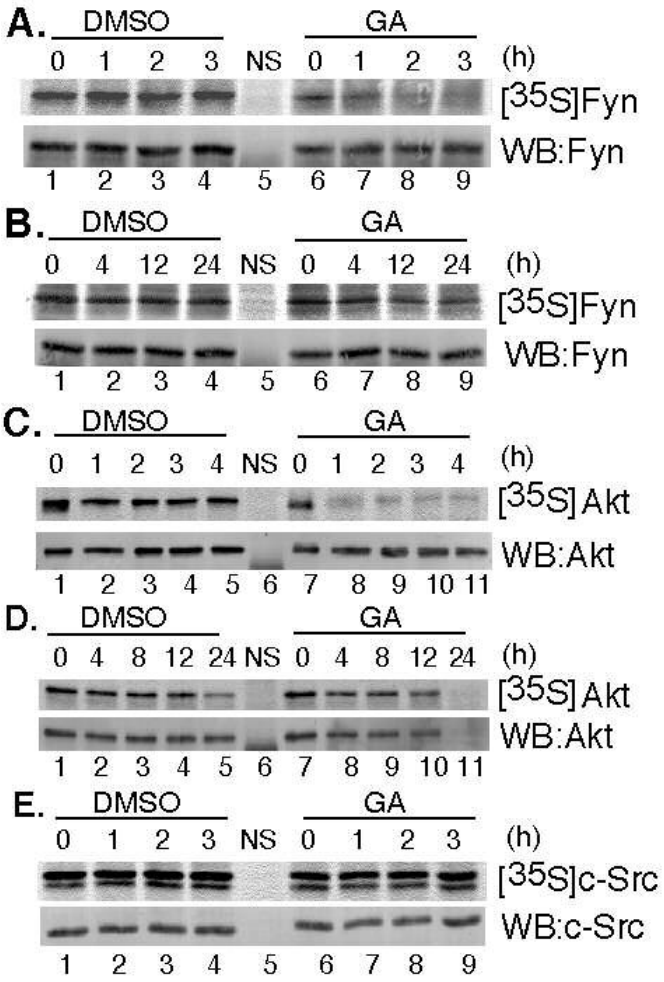

Figure 19. Pulse-chase analyses of the effects of geldanamycin on [^{35}S]-labeled protein kinases in differentiating C2C12 myoblasts. Pulse-chase experiments were carried as described under "Experimental Procedures". Briefly, cells maintained in DM were pulsed with [^{35}S]Met in the presence of DMSO or 5µg/mL geldanamycin (GA) for 30 min, and then chased with fresh DM containing DMSO (A & E: lanes 1-4 and C: lanes 1-5) or geldanamycin (GA; A & E: lanes 6-9 and C: lanes 7-11) for 0 to 4 h as indicated (A, C, & E). Cells maintained in DM were pulsed for 30 min with [^{35}S]Met, transferred to fresh DM for 3hrs, and then treated with DMSO (B: lanes 1-4; and D: lanes 1-5) or 5 µg/mL geldanamycin (GA; B: lanes 6-9; and D: lanes 7-11) for 0 to 24 h as indicated (B & D). Fyn (A & B), Akt (C & D), and c-Src (E) were immunoadsorbed with anti-Fyn, anti-Akt, or anti-c-Src agarose resin and analyzed by SDS-PAGE. [^{35}S]-labeled proteins were detected by autoradiography ([^{35}S]) or western blotting (WB) with kinase specific antibodies. NS: non-immune IgG resin for non-specific binding of proteins (A, B, & E: lane 5, and C & D: lane 6).

geldanamycin. These data suggest that geldanamycin-inhibitable Hsp90 function may not be required for folding and stability of c-Src in differentiating C2C12 myoblasts.

To determine the effect of Hsp90-inhibition on the stability of mature Fyn, and Akt molecules, C2C12 cells were pulse-labeled with [^{35}S]-Met for 30 min, and then subsequently incubated with fresh media for 3 h. Cells were then incubated with or without geldanamycin for 0 to 24 h. The stability of mature [^{35}S]-Fyn appeared to be unaffected in the presence of geldanamycin (Fig 19B), as mature [^{35}S]-Fyn turned over at a rate comparable to that of mature Fyn in control cells. On the other hand, geldanamycin appeared to moderately accelerate the turnover of mature [^{35}S]-Akt, leading to its nearly complete degradation after 24 h, compared to only a 50% reduction of mature [^{35}S]-Akt in control cells at 24 h (Fig. 19D). This result suggests that Hsp90 is not required to maintain the stability of mature Fyn molecules in differentiating C2C12 myoblasts. Furthermore, the gradual geldanamycin-induced depletion of Fyn and Akt from differentiating C2C12 cells appears to be primarily due to the inability of the cells to mature newly synthesized kinase molecules, and thus, replenish mature kinase molecules that are slowly degraded in the cells.

Interaction of Fyn with Hsp90 and Cdc37- Previously, we have demonstrated that the Src-family kinases, Lck and Hck are Hsp90-dependent clients that interact with Hsp90 and its "kinase-specific" co-chaperone Cdc37 (139, 141, 190-192, 253). Therefore, we examined whether Fyn similarly interacts with Hsp90 and Cdc37, and whether this interaction was disrupted by geldanamycin-induced Hsp90 inhibition. C2C12 cells were treated with geldanamycin for 3 h, followed by immunoadsorption of Fyn from cell extracts. Consistent with results obtained with the other Src family

kinases, Hsp90 and Cdc37 were co-adsorbed in a complex Fyn, and inhibition of Hsp90 by geldanamycin resulted in the dissociation of Hsp90 and Cdc37 from Fyn (Fig 20A). These results demonstrate that Fyn interacts directly with Hsp90 and Cdc37. However, in similar experiments carried out with c-Src, we could not reproducibly demonstrate an interaction of Hsp90 with c-Src, while an interaction of Cdc37 with c-Src was consistently observed (data not shown).

The effects of Hsp90 inhibition on the expression of myogenic markers during C2C12 differentiation- As discussed in the introduction, ErbB2, Akt, and Fyn are known to play critical roles in regulating signal transduction pathways during myoblast differentiation. The data presented above indicate that Hsp90 function is required for the maturation and/or stability of these kinases during C2C12 myoblast differentiation. The myogenic markers, MyoD and myogenin, are transcription factors that regulate the expression of muscle-specific genes during myoblast differentiation and the formation of myotubules (166, 182, 186, 200-203, 210, 234, 240). Recent studies indicated that expression of Akt2 is required for myogenin expression, and that the expression of these two proteins correlated with the developmental processes that are involved in myoblast differentiation (160, 166, 168, 186).

Therefore, we examined whether inhibition of Hsp90 function influenced the stability or expression of the myogenic markers, MyoD and myogenin. C2C12 myoblasts constitutively express the myogenic marker MyoD (166, 182, 210, 223). At confluence C2C12 cells were treated (or not) with geldanamycin for periods of 20 h at 0, 15, and 24 h, respectively, after induction of differentiation.

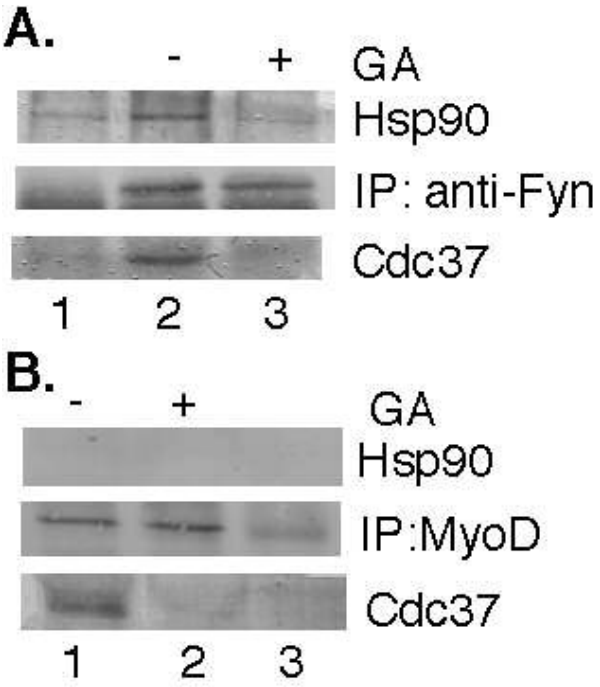

Figure 20. Interaction of Fyn with Hsp90 and Cdc37. Confluent cells maintained in DM for 40 h were treated with DMSO or 5 µg/mL geldanamycin (GA) for 3 h. Cell extracts were prepared as described under "Experimental Procedures". Fyn (A) and MyoD (B) were immunoadsorbed with anti-Fyn and anti MyoD agarose resin, respectively. Proteins were analyzed by western blotting with anti-Hsp90, anti-Fyn, anti-Cdc37, and anti-MyoD antibodies. A, lane 1 & B, lane 3: non-immune IgG resin for non-specific binding of proteins.

While previously published data suggest that MyoD function may be dependent on Hsp90 (241, 242), geldanamycin-induced inhibition of Hsp90 had little, if any, effect on the amount of MyoD expressed in differentiating C2C12 cells (Fig. 20 and 21). Interestingly, Cdc37 was found to co-immunoadsorb with MyoD, while Hsp90 was not detectable on western blots of the co-immunoadsorption experiments (Fig 20B). However, the interaction of MyoD with Cdc37 was disrupted by geldanamycin, suggesting that the interaction of Cdc37 with MyoD may be an Hsp90-dependent event.

Myogenin expression is dependent upon the functions of Akt1, Akt2, and MyoD (166). The expression level of myogenin was increased in C2C12 cells, 24 h after induction of differentiation (Fig. 21A, DMSO). Addition of geldanamycin at the beginning or after 15 h of the induction of differentiation blocked myogenin expression (Fig 21A, GA). Addition of geldanamycin to cells 24 h after induction of differentiation, only partially suppressed myogenin expression, as myogenin was already being expressed prior to treatment of cells with geldanamycin. Geldanamycin-induced suppression of myogenin expression correlated with geldanamycin-induced depletion of Akt1 and Akt2 from the differentiating C2C12 cells (Fig. 21B): an observation consistent with previous work indicating that the function of both Akt1 and Akt2 is required for the induction of myogenin expression (166). Thus, the data indicate that Hsp90 function is essential for the expression of the myogenic regulatory factor, myogenin, which is critical for myoblast differentiation.

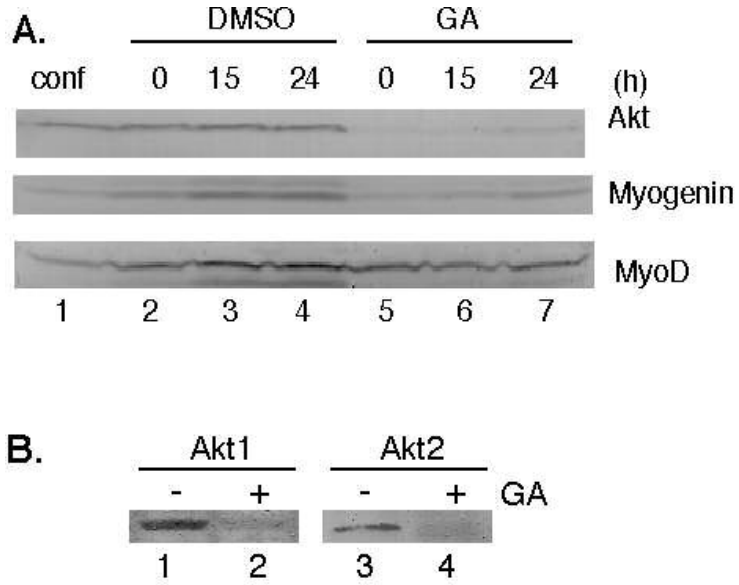

Figure 21. Effects of geldanamycin on the expression of myogenin in C2C12 cells. **(A)** Confluent C2C12 cells were treated with DMSO or 5 µg/mL geldanamycin (GA) for 20 h at 0, 15, and 24 h after induction of differentiation. (B) Cells were treated with DMSO or 5 µg/mL geldanamycin for 24 h after maintained in DF for 48 hr. Cells were lysed directly with SDS sample buffer and then analyzed by western blotting with anti-Akt (detecting both Akt1 & Akt2), anti-Akt1, anti- Akt2 anti- MyoD and anti-myogenin specific antibodies.

DISCUSSION

Our data indicate that Hsp90 function is essential for the survival and differentiation of C2C12 myoblasts. Previously, we have demonstrated a temporal and spatial requirement for Hsp90 function within somatic cells of zebrafish, which is necessary for the formation and possibly the survival of eng-2-expressing muscle pioneer cells and possibly other striated muscle fiber types (254). Furthermore, functional ErbB2, Akt and Fyn have been demonstrated to be required for myoblast differentiation and for the survival of mature muscle cells and muscle satellite cells (155, 156, 187, 205, 208, 231, 232, 234, 238, 255). Treatment of differentiating C2C12 cells with geldanamycin caused the depletion of ErbB2, Akt and Fyn from the cells. Thus, multiple signal transduction pathways are impaired upon treatment of differentiating C2C12 cells with geldanamycin, and consistent with the requirement of these kinases for cell survival, inhibition of Hsp90 with geldanamycin in C2C12 cells appeared to lead to the induction of apoptosis.

The selective tumoricidal effects of ansamycins are believed to be due to their ability to interfere with Hsp90-dependent pro-growth and anti-apoptotic signaling pathways that fuel the proliferation of cancer cells, leading to the arrest of the cell cycle and apoptosis (110, 180, 195, 243, 246). The cytotoxic effect of geldanamycin on differentiating myoblasts suggest that geldanamycin can also adversely affect the viability of cells that have escaped from the cell cycle, but are still dependent on anti-apoptotic or pro-growth signaling pathways for cell survival.

Cancer cells that overexpress ErbB2 are highly susceptible to the tumoricidal effects of geldanamycin (173, 184, 188). ErbB2 is also the target for

Trastuzumab/Herceptin, a novel and effective antibody-based therapy used for the treatment of mammary carcinomas. Mice specifically expressing a mutated form of ErbB2 in ventricular cardiomyocytes, develop a severe dilated cardiomyopathy (207). Compared to wild-type myoblasts, ErbB2-null myoblasts were also found to be much more sensitive to apoptotic cell death during differentiation (208). Cardiomyopathies have also been observed to develop in a proportion of patients treated with Trastuzumab/Herceptin (205, 209). Thus, ErbB2 appears to play an important role in maintaining the differentiation, function or survival of cardiomyocytes (206).

Activation of Akt in cardiomyocytes also leads to an increase in cell size and prevents apoptosis (153, 154, 157, 159, 163). Mice overexpressing a constitutively active Akt mutant in the heart show improved cardiac function with concomitant cardiac hypertrophy (157, 163). Thus, the ability of geldanamycin to deplete muscle cells of both ErbB2 and Akt suggests that certain regimens of geldanamycin treatment could potentially have adverse effect on cardiac muscle and cardiovascular function.

In addition to their roles in regulating the growth and survival of cardiac muscle cells, ErbB2, Akt and Fyn similarly regulate the growth and survival of skeletal muscle cells (153, 155, 158, 161, 162, 208, 234, 238, 255). Overexpression or hyperactivation of these kinases leads to hypertrophy of skeletal muscle, while the down-regulation or inhibition of their function leads to skeletal muscle atrophy. Thus, loss of skeletal muscle mass may also be a possible side effect of certain regimens of geldanamycin.

However, increases in cell mass can also have adverse effects on cardiac function. Hypertrophy of cardiac cells induced by an increased load on the heart as a consequence of hypertension, ultimately leads to cell death and congestive heart failure (159, 205, 216,

256). Loss of cardiac tissue occurs, at least in part, through induction of apoptosis. Thus, therapies designed to inhibit Hsp90 function with the aim of preventing the hypertrophy of smooth muscle cells in the vasculature (164, 167, 211, 214, 257), and thus the development of hypertension, or the consequent increase in cardiac muscle cell mass that occurs in response to elevated blood pressure may have some benefit in the deterrence of events that lead to congestive heart failure.

The activity of the Src-family kinase Fyn is required for fusion of differentiated muscle cells and the survival of differentiated myotubules (234, 238, 239). Like its Srє family relatives, Lck and Hck, Fyn was demonstrated to interact with Hsp90 and Cdc37, and its protein levels were depleted from cells treated with geldanamycin. These results identify Fyn as a new Hsp90-dependent client. Similar to Lck and Hck (191, 253), geldanamycin-induced inhibition of Hsp90 function led to the depletion of Fyn levels in differentiating C2C12 cells due to the inhibition of its folding and maturation: inhibition of Hsp90 function by geldanamycin had little effect on the rate of turnover of mature Fyn molecules. Thus, loss of functional Fyn from differentiating myoblasts also likely contributes to the subsequent death of these cells in the presence of geldanamycin.

Curiously, geldanamycin treatment of differentiating C2C12 cells had no affect on the stability of either newly synthesized or mature forms of c-Src. Similar to studies carried out in the 1980's in other cell lines (258, 259), we failed to detect an interaction between Hsp90 and c-Src in differentiating C2C12 cells. However, an interaction between newly synthesized c-Src and Hsp90 has been reconstituted *in situ* in reticulocyte lysate (260), and B.T. Scroggins, S.D. Hartson & R.L. Matts, unpublished observations), and Hsp90 is required for the activity of c-Src expressed in yeast (261). On the other

hand, while Bijlmaker and Marsh (262) observed that geldanamycin led to the complete destabilization of newly synthesized c-Src overexpressed in transfected 3T3 fibroblasts, we observed that geldanamycin had no effect on the stability of either newly synthesized or mature c-Src endogenously expressed in C2C12 cells. Bijlmaker and Marsh (262) similarly observed that geldanamycin had no effect on the stability of mature c-Src expressed by transfection in 3T3 cells. Cdc37 was consistently observed to co-immunoadsorb with c-Src from C2C12 extracts, suggesting that Cdc37 may be able to interact with and/ or stabilize c-Src independently of Hsp90 in C2C12 myoblasts. While our failure to document an interaction of Hsp90 with c-Src might result from the instability of their interactions in the present of detergents, the absence of any effect of geldanamycin on c-Src stability in C2C12 cells suggests that the dependence of c-Src on Hsp90 for its maturation and stability may be dependent upon the particular cell type in which it is expressed.

Myogenin is one of the earliest molecular markers for commitment to myogenic differentiation, and the transcriptional activity of myogenin, in conjunction with other members of the MyoD family of transcription factors, is required for the expression of muscle-specific genes induced during muscle differentiation (201-203, 263, 264). Akt2 activity is required for the trans-activation of the transcriptional activity of MyoD-MEF2, and the upregulation of myogenin expression in differentiating muscle cells (165, 166, 186, 210). The ability of geldanamycin to deplete C2C12 cells of Akt2 likely explains the capacity of geldanamycin to block the expression of myogenin. The mechanism by which the absence of Akt2 blocks the trans-activation of MyoD-MEF2 remains to be determined.

MyoD has been postulated to be a target of Hsp90 (241, 242). However, this hypothesis is based on in vitro studies demonstrating the ability of a C-terminal fragment of Hsp90 to activate the DNA-binding activity of a fragment MyoD (241, 242). No interaction of Hsp90 with the MyoD present in differentiating C2C12 cells was observed using antibody pull-down assays, and geldanamycin treatment had no effect on the level of expression of MyoD in differentiating C2C12 cells. These observations suggest that MyoD stability is not dependent on its interaction with Hsp90. However, an interaction of MyoD with Cdc37 was observed, and this interaction was inhibited by geldanamycin. A functional interaction of the androgen receptor with Cdc37 has been shown to be required for its transcription activation, in addition to its interaction with Hsp90 (265). Reverse transcriptase has also been identified as a non-kinase client of Cdc37 [reviewed in (35)].

The Hsp90-dependent interaction of Cdc37 with MyoD suggests a number of potential mechanisms by which geldanamycin could indirectly inhibit the function of MyoD and block myogenin expression. MyoD is a phospho-protein and changes in its phosphorylation state have been correlated with its hetero-dimerization with other factors and its transcriptional activation (266-271). Thus, geldanamycin-induced inhibition of any of these Hsp90-dependent protein kinases could block MyoD's trans-activation or alter its function[reviewed in(13, 101)]. Furthermore, the kinase-binding activity of Cdc37 requires its phosphorylation by casein kinase 2 (272), an Hsp90-dependent kinase (273). In the absence of phosphorylation, Cdc37 may not be capable of binding MyoD, or MyoD-associated Cdc37 may not be able to bind kinase and act as a scaffold to facilitate MyoD phosphorylation.

The data presented in this report demonstrate that the inhibition of Hsp90 function leads to depletion of ErbB2, Akt1, Akt2 and Fyn from C2C12 myoblasts, and thus the impairment of a number of signal transduction cascades critical for orchestrating the differentiation C2C12 cells. This study indicates, not unexpectedly, that the impairment of the function of no single Hsp90-dependent protein client can necessarily account the overall effect of geldanamycin on a cell's physiological state. In addition, further studies are clearly required to determine how inhibition of Hsp90-dependent signal transduction cascades interferes with trans-activation of transcription factors in differentiating muscle cells, as well as in other cell systems.

CHAPTER V

Interdomain interactions regulating the activation of the heme-regulated eIF2α kinase

Abstract

The heme-regulated inhibitor of protein synthesis (HRI) regulates translation in reticulocytes through its ability to phosphorylate the α-subunit of the eukaryotic initiation factor 2 (eIF2). The structure of HRI consists of an N-terminal heme-binding domain (NT-HBD) followed by its kinase domain. While HRI is best known for its activation in response to heme deficiency, we recently showed that the binding of NO and CO to the NT-HBD of HRI activates and suppresses the activity of HRI, respectively. To further study the structure/function relationships between the NT-HBD and the catalytic domain of HRI (HRI/ΔHBD), we examined the effect of hemin, NO and CO on the interactions of the two domains expressed separately in reticulocyte lysate. In the presence of hemin, HRI/ΔHBD interacted with His-tagged NT-HBD, and NO, but not CO, disrupted the domain-domain interaction. When transferred to heme-deficient lysate, the interaction of the HRI/ΔHBD with His-tagged NT-HBD decreased by approximately 75%. In heme-deficient lysate, the interaction between the two domains was disrupted by NO, but was stabilized by CO. A deletion mutant of HRI (ΔH-HRI), lacking a helix inside the

NT-HBD predicted to be structurally equivalent to the H-helix of hemoglobin, was markedly less sensitive to heme-induced inhibition. Furthermore, mutant NT-HBD, lacking the H helix did not bind HRI/ΔHBD, suggesting that the deleted region is critical for regulation of HRI activity. HRI/ΔHBD and ΔH-HRI, unlike wild type HRI, underwent nearly complete transformation into its active form: a result consistent with a change in the heme-binding activity of these two mutant constructs. Thus, the kinase activity of HRI appears to be regulated through an interaction between its NT-HBD and its catalytic domain, as the activation of HRI by heme deficiency or NO correlated with an alteration in the interaction between these two domains.

Introduction

The heme-regulated inhibitor of protein synthesis (HRI) is a member of a family of protein kinases that regulate initiation of protein synthesis in eukaryotic cells through the phosphorylation of the α-subunit of eukaryotic initiation factor-2 (eIF2α) [reviewed in (274, 275)]. The primary function of HRI is thought to be the coordination of the synthesis of globin chains in reticulocytes with heme availability. Heme is required for the assembly of α- and β-globin chains into hemoglobin. Thus, a deficiency of heme induces the activation of HRI, which subsequently phosphorylates the α-subunit of eIF2, leading to the inhibition of polypeptide chain initiation and the arrest of protein synthesis. This prevents the accumulation of unassembled globin chains, the aggregation of which is toxic to red blood cells.

HRI is a multidomain protein that appears to contain two distinct heme-binding sites (276-278). The heme-binding site that is thought to regulate HRI activity in

response to changes in heme concentration, has been tentatively localized to the "kinase insertion domain" of HRI (277). The N-terminal domain of HRI contains the second heme-binding site (NT-HBD), which consists of approximately 165 amino acids (277, 278). This domain contains the heme, which is stably bound and co-purifies with HRI (276-278). The NT-HBD appears to be the site for the binding of the diffusible gas second messengers, nitric oxide (NO) and carbon monoxide (CO), which enhance and suppress the activation of HRI, respectively, as HRI lacking most of the NT-HBD has kinase activity that does not respond to the presence of NO or CO (279). However, while HRI lacking most of the NT-HBD does not purify as a hemoprotein, its kinase activity responds to the presence of heme, suggesting the existence of a second heme-binding site (276, 277).

The sequence alignment of the NT-HBD to globins and preliminary spectroscopic analysis have suggested that histidine is the axial ligand of the heme-binding site in the NT-HBD (278). The heme bound NT-HBD forms a six-coordinate NO complex (278, 280). The formation of this six-coordinate NO-Fe-His complex differs from the complex formed in the prototypical NO-responsive sensor protein, soluble guanylate cyclase. In soluble guanylate cyclase, NO-binding to the heme iron induces cleavage of the iron-histidyl bond (281). The formation of a five-coordinate NO complex is thought to trigger for the activation of guanylate cyclase (281). Since the iron-histidyl bond is retained in the NO adduct of the NT-HBD of HRI, we have proposed that changes in the conformation of amino acid residues adjacent to the NO binding site (the distal-heme ligand), not cleavage of the axial histidine ligand at the proximal side of the heme-binding site, may be crucial for the NO-induced activation mechanism in HRI (280).

Recently, we prepared point mutants substituting Ala for His at each of the seven histidine residues present in the NT-HBD of rabbit HRI. Sequence alignments indicated that two of the residues (His^{78} and His^{123}) were invariant from zebrafish to human (282). UV-visible absorption, CD, EPR and resonance Raman spectroscopy of purified recombinant wild-type and mutant NT-HBDs expressed in *E. coli*, indicated that His^{78} and His^{123} are likely the two crucial residues for the ligation of heme within the NTD of HRI (282). On the other hand, studies with recombinant full length HRI suggest that Cys may be an axial heme ligand, and that NO activation may involve the formation of a five-coordinate NO-heme (283). However, there are limitations to the conclusions one can draw from studies utilizing recombinant HRI, as the protein appears to be a heterogeneous mixture of molecules in different states of phosphorylation, and the phosphorylation state of HRI is known to alter its heme-binding affinity(284 -287).

In this study, we examined the effect of heme deficiency, NO and CO on the interaction between the NT-HBD and the catalytic domain of HRI from which the entire NT-HBD was deleted (HRI/ΔHBD). The results indicate that stimuli, such as heme deficiency and NO that activate HRI, disrupt the interaction between the NT-HBD and HRI/ΔHBD, while the interaction between the two domains is retained in heme-replete lysate and in the presence of CO. Thus, we propose that HRI activation is regulated by interdomain interactions between the NT-HBD and the catalytic domain, and that effectors of HRI activation induce a conformational change within the NT-HBD of HRI that alters its inhibitory interaction with the catalytic domain leading to the activation of its kinase activity.

Experimental Procedures

Construction of mutants. Mutant HRI (HRI/ΔHBD), representing sequences starting at residue 158 to its C-terminus, was cloned into a modified pSp64T plasmid using a StyI restriction site, and mutant HRI (ΔH-HRI), representing sequences from HRI lacking the deleted region (residues 116 to 158 from N-terminus of HRI, ΔH), was cloned by PCR into a modified pSp64T plasmid using StyI and PmeI restriction enzyme sites, as described previously (45). Mutant NT-HBD (NT-HBD/ΔH) was cloned by PCR into a modified pSp64T plasmid using SalI and PmeI restriction enzyme sites. NT-HBD and wild type HRI plasmids were described previously (45). N-terminally His-tagged or non-His tagged versions of each mutant were made as described previously (45, 278).

Analysis of the interaction between the NT-HBD or NT-HBD/ΔH and the HRI/ΔHBD in reticulocyte lysate. HRI constructs were synthesized and radiolabeled by coupled transcription/translation in nuclease-treated rabbit reticulocyte lysate in the presence of [^{35}S]Met for 30 min at 30ºC, followed by a 10-min incubation after the addition of aurintricarboxylic acid (60 μM final) to stop further initiation of protein synthesis(138, 279) . Aliquots (3 μl) of each construct were transferred to untreated heme-deficient reticulocyte lysate protein synthesis mixtures (22 μl), and then incubated for an additional 45 min. For heme-replete samples, reaction mixtures were supplemented with hemin (10 μM) for 15 min and then treated with the NO generator NOC-9 (1 mM) for 10 min, or gassing with 100% carbon monoxide (CO) for 5 min on ice (279). His- tagged constructs were immunoadsorbed with anti-His_5 monoclonal antibody (Qiagen) bound to anti-mouse agarose resin at 4ºC for 1 to 2 h as previously described (28, 279). Samples were then washed 4 times with wash buffer (10 mM PIPES

pH 7.2, 150 mM NaCl) and analyzed by SDS-PAGE. All HRI constructs were visualized by autoradiography.

Assay of the kinase activity of wild-type HRI and mutant ΔH-HRI. HRI was synthesized *de novo* in reticulocyte lysate by coupled transcription/ translation and matured in hemin-supplemented (10 μM) or heme-deficient rabbit reticulocyte lysate for 60 min, or matured in heme deficient RRL for 45 min followed by incubation in the presence 10 μM hemin for 15 min as described above. His-tagged wild-type and ΔH-HRI were immunoadsorbed with anti-His-tag antibody resin, and washed as described above. Kinase activity was assayed by incubation of each sample with eIF2 for 5 min at 30ºC in the presence of [γ-^{32}P]ATP, followed by SDS-PAGE and analysis by autoradiography, as described previously (138).

Analysis of the transformation of wild-type and mutant HRIs. [^{35}S]-labeled wild-type HRI, ΔH-HRI and HRI/ΔHBD were synthesized for 30 min as described above. The samples were matured at 30ºC for 0 to 180 min in heme-deficient reticulocyte lysate. Proteins were immunoadsorbed with anti-His tagged antibodies and washed as described above, and then analyzed by SDS-PAGE on 7% gels, transferred to PVDF membranes and visualized by autoradiography (138).

Results and Discussion

Effects of heme deficiency, NO and CO on the interaction of the NT-HBD and the catalytic domain (HRI/ΔHBD) of HRI. Although the activation of HRI has been know to be regulated by heme for over two decades, almost nothing is known about the physical basis behind the mechanism of this phenomenon. Currently, the binding of hemin is

postulated to repress the activity of HRI through the induction of an intermolecular disulfide bond between subunits within dimeric HRI (288, 289). Similarly, while the physical characteristics of the coordination complex formed between NO and the NT-HBD and full length recombinant HRI have been investigated (279, 280, 282, 283), little is known about how the binding of NO modulates the activation of HRI. However, our previous studies have shown that the NT-HBD expressed in trans had the capacity to modulate NO stimulation of the kinase activity of a HRI mutant (HRI/Met3) from which most of the NT-HBD had been deleted (279). Furthermore, the HRI/Met3 mutant was only about 50% as active as wild-type HRI, while being less responsive to inhibition by heme (277, 279). However, the activity of the HRI/Met3 mutant was markedly enhanced when the NT-HBD was expressed in trans (279). These observations suggest that the NT-HBD potentially has both positive and negative effector functions with respect to regulation of HRI's kinase activity.

To test the whether conditions that activate HRI alter interactions between its NT-HBD of HRI and its catalytic domain, we constructed a HRI mutant from which the entire coding sequence of the NT-HBD was deleted (HRI/ΔHBD). His-tagged NT-HBD and HRI/ΔHBD were then synthesized by coupled transcription/ translation in reticulocyte lysate and mixed. The samples were then diluted into heme-deficient reticulocyte lysate to allow the kinase domain to undergo Hsp90-dependent maturation (279). Samples were then left untreated or treated with hemin, the NO-generator NOC-9, CO, or hemin in conjunction with NOC-9 or CO, and the ability of anti-His tag antibody to co-immunoadsorb HRI/ΔHBD with His-tagged NT-HBD was determined.

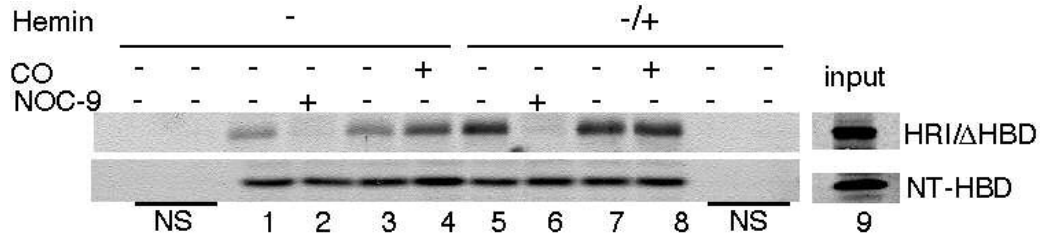

Figure 22. Effects of heme, NO, and CO on the interaction between the NT-HBD and the catalytic domain (HRI/ΔHBD) of HRI. [^{35}S]-labeled His-tagged NT-HBD and untagged HRI/ΔHBD were synthesized in reticulocyte lysate, mixed and matured in heme-deficient lysate as described under "Experimental Procedures". Subsequently, samples were incubated in the absence (lanes 1-4) or presence (lanes 5-8) of hemin for 15 min, and then left untreated treated (lanes 1, 3, 5, and 7), or were treated with 1 mM NOC-9 (lanes 2 and 6) for 10 min at 30ºC, or with CO (lanes 4 and 18) for 5 min on ice. His-tagged NT-HBD was immunoadsorbed and samples were analyzed for co-adsorbed HRI/ΔHBD by SDS-PAGE and autoradiography. NS: Protein non-specifically bound to non-immune IgG resin from heme-deficient (left side) and heme-replete (right side) lysate. Input: Represents the synthesized protein used in the experiment.

As shown in Fig 22, addition of hemin markedly enhanced the interaction of NT-HBD with HRI/ΔHBD (lane 1 versus 7). The presence of NO, on the other hand, disrupted the interaction between the NT-HBD and HRI/ΔHBD regardless of the presence of hemin (lanes 2 and 6). In contrast, while CO had little further effect on the hemin-induced stabilization of the two domains in hemin-supplement lysate (lane 7 versus 8), the presence of CO caused a perceptible increase in the interaction between the two domains in heme-deficient lysate (lane 3 versus 4). Thus, conditions that cause activation of HRI's kinase activity, heme deficiency and the presence of NO, altered or disrupted the interaction between HRI's NT-HBD and its catalytic domain, while conditions that repress HRI activation (heme sufficiency or CO) stabilized the interaction. It is likely that the altered interaction between the two domains is a result of a conformational change induced by the absence of reversible heme-binding to HRI's second heme-binding site, or the coordination of NO by the NT-HBD, and this altered conformation presumably modulates the activation of HRI. Furthermore, conditions that promote the interaction between the NT-HBD and kinase catalytic domain (HRI/ΔHBD) presumably stabilize a conformation that inhibits HRI's catalytic activity.

Deletion of the "H-helix" region generates hyperactive HRI. Sequence analysis of HRI indicated that its contains an Erythrocruorin family (globin super-family) signature spanning amino acid residues 66 to 89 (278), and that residues 11-118 in the NT-HBD of HRI have significant sequence similarity to amino acids 16-120 in mammalian α-globins. However, while the analysis suggest that the NT-HBD of HRI may have evolved from the second intron of the α-globin gene (278), the finding that

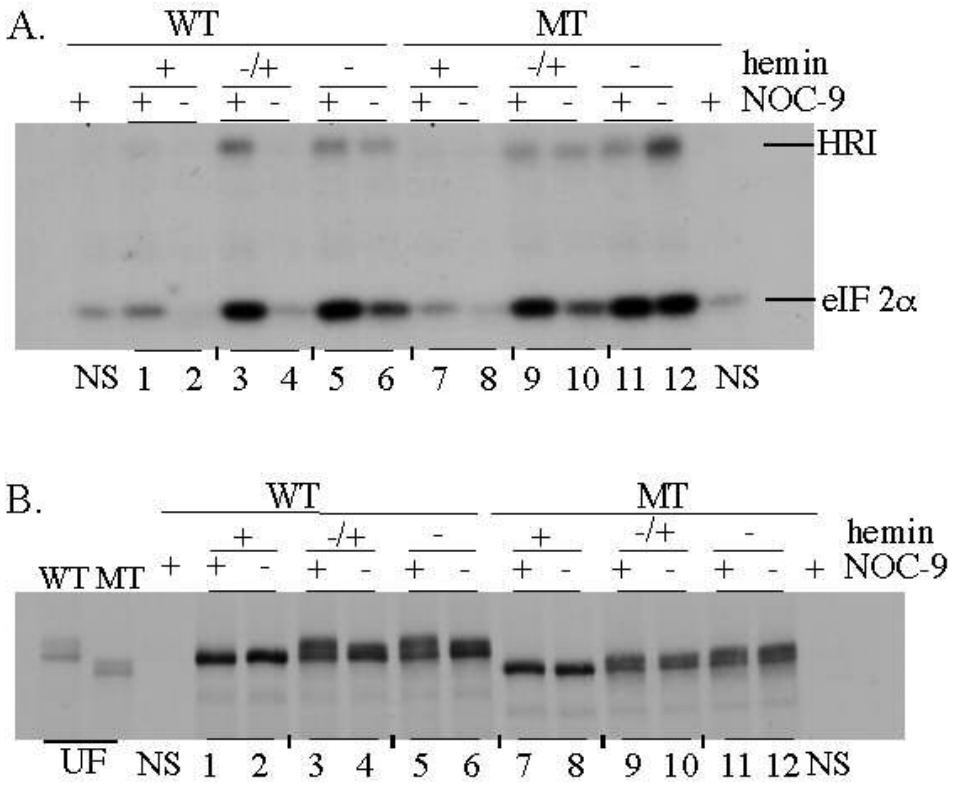

Figure 23. Comparison of the regulation of the kinase activity of wild type HRI and mutant ΔH-HRI. [^{35}S]-labeled His-tagged HRI and His-tagged ΔH-HRI were synthesized in reticulocyte lysate and matured in heme-deficient (lanes 3- 6, 9-12) or heme-replete lysate (lanes 1, 2, 7, and 8) for 45 min as described under "Experimental Procedures". Samples were then matured for an additional 15 min with no additions or with the addition (lanes 3, 4, 9, and 10) of 10 μM hemin to the heme-deficient lysate (heme-supplemented). Heme deficient (-), or heme-supplemented (-/+), or heme-replete (+) reticulocyte lysates were then treated with 1 mM NOC-9 (lanes 1, 3, 5, 7, 9, and 11) or buffer (control: lanes 2, 4, 6, 8, 10, and12) for 10 min at 30ºC. His-tagged HRI and ΔH-HRI were immunoadsorbed and assayed for eIF2α and autokinase activity for 5 min at 30ºC as described under the "Experimental Procedures". Samples were analyzed by SDS-PAGE and autoradiography. (A) Autoradiogram of [^{32}P]-eIF2α and [^{32}P]-HRI. (B) Autoradiogram of immunoadsorbed [^{35}S]-HRI. NS: Kinase activity of protein non-specifically bound to non-immune IgG resin from heme-deficient lysate treated with 1 mM NOC-9. WT: wild-type HRI. MT: mutant ΔH-HRI. UF: Sample of unfractionated lysate before the immunoadsorption of [^{35}S]-HRI in heme-deficient reticulocyte lysate.

His^{123} may be one of the axial heme-binding ligands (282), suggests that the tertiary fold of the NT-HBD may be different from that of globin chains, as H^{123} is predicted to be part of what would be equivalent to the H-helix of globins: a helix located outside the heme-binding core of globins (278).

To further investigate the relationship between the mechanisms by which HRI's catalytic activity is repressed by heme and activated by NO, we constructed a mutant of HRI (ΔH-HRI) lacking the putative H-helix and linker region (amino acids 116-157) connecting the NT-HBD to the catalytic domain. We hypothesized that the deletion of this region, which contains the domain's putative distal heme-binding ligand, would alter the response of HRI's catalytic domain to NO. While mutant ΔH-HRI was found to have detectable eIF2α kinase activity (Fig. 23A: lane 8) compared of wild-type HRI (Fig. 23A:lane 2) when the newly synthesized protein was maintained in the presence of hemin, the transformation of both wild-type (Fig. 23B: lanes 1 and 2) and ΔH-HRI (Fig. 23B: lanes 7 and 8) into an NO-activatable kinase was effectively blocked by heme (compare to NS in Fig. 23A: activity of non-specifically bound NO-activatable kinase activity). However, after the proteins were incubated in heme-deficient lysate to allow them to undergo Hsp90-dependent "transformation" into active kinases (138), the activity of wild-type HRI was fully repressed by the addition of hemin (Fig. 23A: lane 4), while the activity of ΔH-HRI was not (Fig. 23A: lane 10). On the other hand, both repressed wild-type HRI (Fig. 23A: lane 3) and repressed ΔH-HRI (Fig. 23A. lane 9) were equally responsive to activation by NO.

Distinct differences in the properties of ΔH-HRI were also noted in heme-deficient lysate (Fig. 23). ΔH-HRI (Fig 23A: lane 12) was hyperactivated in heme-

deficient lysate compared to wild-type HRI (Fig. 23A: lane 6). Furthermore, the already hyperactivated ΔH-HRI was only marginally simulated by NO in heme-deficient lysate (Fig. 23A: lane 11) compared to unstimulated ΔH-HRI (Fig. 23A: lane 12), such that the activities of unstimulated and NO-stimulated ΔH-HRI were comparable to the activity of wild type HRI that was hyperactivated by NO stimulation in heme-deficient lysate (Fig. 23A: lanes 5). Therefore, while ΔH-HRI maintained characteristics of wild type HRI relative to the ability of hemin to inhibit its Hsp90-dependent "transformation" into an active, regulatable kinase, the activity of "transformed" ΔH-HRI was much less sensitive to repression by hemin. These results suggest that the deleted region is important for attenuating activation of HRI in response to heme-deficiency, as the mutant protein is hyperactivated to a level comparable to that induced by the presence of NO.

The "H-helix" and linker region of the NT-HBD are required for the stable interdomain interactions induced by the presence of hemin. The results described above suggest that the deletion of amino acids 116-157 ("H-helix" and linker region) impairs the ability of hemin to repress the activity of transformed ΔH-HRI. To test this hypothesis, a His-tagged NT-HBD mutant was constructed from which amino acids 116-157 were deleted (NT-HBD/ΔH). We then examined the ability of the mutant to interact with transformed HRI/ΔHBD in trans in the presence or absence of hemin. As demonstrated previously in Figure 22, less HRI/ΔHBD was co-adsorbed with His-tagged NT-HBD from heme-deficient lysate (Fig. 24, lane 1) after its transformation, than from lysate where the activity of transformed HRI/ΔHBD had been repressed by the addition of hemin (Fig. 24,lane 2). In contrast, little detectable HRI/ΔHBD was co-adsorbed with

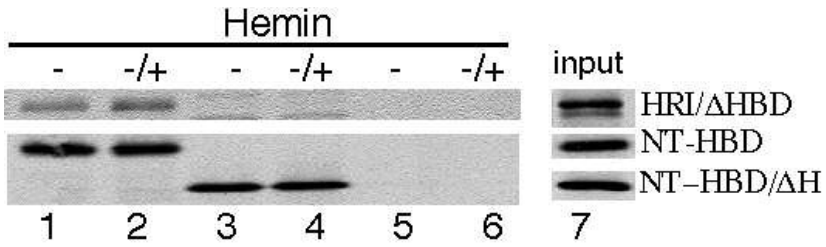

Figure 24. Interaction of the NT-HBD/ΔH mutant with the catalytic domain (HRI/ΔHBD) of HRI. [^{35}S]-labeled His-tagged NT-HBD, His tagged NT-HBD/ΔH and untagged HRI/ΔHBD were synthesized in reticulocyte lysate as described under "Experimental Procedures". The His-tagged constructs were mixed with lysate containing HRI/ΔHBD and matured in heme-deficient lysate for 45 min. Subsequently, samples were incubated in the absence (lanes 1, 3, and 5) or presence (lanes 2, 4, and 6) of 10 μM hemin for 15 min. His-tagged NT-HBD and NT-HBD/ΔH were immunoadsorbed, and samples were analyzed for co-adsorbed HRI/ΔHBD by SDS-PAGE and autoradiography. Protein non-specifically bound to non-immune IgG resin from heme-deficient (lanes 5) and heme-supplemented (lanes 6) lysate. Input: Represents the synthesized protein used in the experiment.

the NT-HBD/ΔH mutant in the presence (Fig. 24, lane 4) or absence (Fig. 24, lane 3) of hemin. Thus, amino acids 116-157 appear to either have a direct functional role in the mechanism by which hemin modulates the interaction of the NT-HBD with the catalytic domain of HRI, or these amino acids are required to maintain the structure of the portion of the NT-HBD that mediates the ability of hemin to modulate the interaction between these two domains.

The ability to bind hemin also appears to attenuate the "transformation" of HRI in heme-deficient lysate (138, 277). In previous studies, we have observed that the transformation of wild type HRI is suppressed in some manner with only about 50% of newly synthesized HRI undergoing transformation when incubated for prolonged times in heme-deficient lysate (138). In these lysates [^{35}S]-HRI migrates as a doublet upon SDS-PAGE with the band exhibiting the fastest migration rate co-migrating with newly synthesized untransformed HRI, and the band exhibiting the slowest electrophoretic mobility representing transformed HRI. Little if any hyperphosporylated HRI, which exhibits an even slower electrophoretic mobility than transformed HRI (41, 129, 142), is observed to be generated during the incubations. Hyperphosphorylated HRI is refractory to inhibition induced upon the addition of hemin (284-287).

Since the data described above suggested that the NT-HBD likely plays a role in the mechanism through which hemin represses the activity of HRI, we examined the electrophoretic properties of ΔH-HRI and HRI/ΔHBD upon their prolonged transformation in heme-deficient lysate. Wild-type HRI, ΔH-HRI and HRI/ΔHBD were observed to undergo transformation at distinctly different rates (Fig. 25). After 90 and 180 min of incubation under heme-deficient conditions, 35 and 75% of wild type HRI

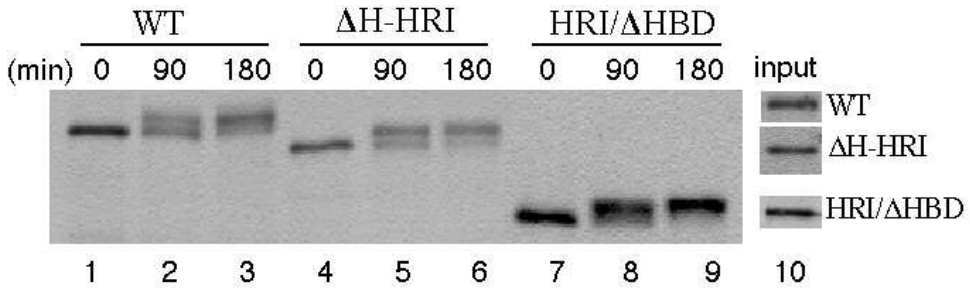

Figure 25. Comparison of the rate of "transformation" of wild type HRI, ΔH-HRI and HRI/ΔHBD. [^{35}S]-labeled His-tagged wild type HRI, ΔH-HRI and HRI/ΔHBD**.** were synthesized in reticulocyte lysate as described under "Experimental Procedures", and then matured and transformed in heme-deficient lysate for 0 (lanes 1, 4, and 7), 90 (lanes 2, 5, and 8) or 180 (lanes 3, 6, and 9) min. Samples were placed on ice at the times indicated in the figure, and then immunoadsorbed and analyzed by SDS-PAGE and autoradiography. WT: wild-type HRI (lanes 1, 2, and 3). ΔH-HRI:mutant HRI (lanes 4, 5, and 6). ΔH-HRI:mutant HRI (lanes 4, 5, and 6). HRI/ΔHBD:mutant HRI (lanes 7, 8, and 9). Input: Represents the synthesized protein used in the experiment.

exhibited the slower electrophoretic mobility diagnostic of kinase "transformation" (see (138)). In comparison, 70 and 85% of ΔH-HRI and 85 and 100% of HRI/ΔHBD had undergone transformation after 90 and 180 min of incubation respectively (Fig. 25). These data suggest that an intact and functional NT-HBD is essential for the mechanism by which HRI maturation and transformation is attenuated in heme-deficient lysate.

Overall, our data suggests that the mechanism by which HRI is activated by heme-deficiency and its mechanism of activation by NO have a feature in common: the altered interaction of HRI's NT-HBD with its catalytic domain. While the interaction between the domains is suppressed in heme-deficient lysate (Fig. 22), it is disrupted by NO, which correlates with the enhanced activation of HRI observed in the presence of NO. On the other hand, the deletion of the amino acids 116-157 disrupted the interaction between the two domains, and no enhanced activation of the ΔH-HRI mutant was observed in heme-deficient lysate in the presence of NO (Fig. 23A). Thus, the degree of activation of HRI or the ΔH-HRI mutant correlates with the interaction, or lack thereof, of the NT-HBD with the kinase domain of HRI. Consistent with this hypothesis the ability of CO to suppress the activity of HRI (279) correlates with its ability to stabilize the interaction between the two domains (Fig. 22).

The data further implies that after HRI's transformation and activation in heme-deficient lysate, HRI activity is regulated by conformational changes that are induced by altered interactions between HRI's NT-HBD and its catalytic domain, and that HRI's "H-helix"-linker region plays an important structural or functional role in the regulation of this interaction These changes could be the result of: 1) HRI's second heme-responsive binding site being comprised of the interface between the NT-HBD and elements of

HRI's catalytic domain, possibly elements present in the "H-helix" and in the kinase "insertion-sequence" as proposed in earlier studies (277); or 2) the reciprocal interactions between the NT-HBD and the second heme-responsive binding site modulating heme-binding affinity of the site coordinately with the affinity of the interaction between the two domains. Thus, agents (e.g., NO) or mutations (e.g., ΔH-HRI) that activate HRI and inhibit the interaction of the NT-HBD with the catalytic domain may coordinately suppress the heme-binding affinity of HRI's second binding site. Alternately, conformational changes induced by dissociation of heme from the second heme-binding site may alter the strength of the interaction between the two domains. Thus the data presented in this report give new insight into the mechanism of regulation of HRI, and indicate that HRI's kinase activity is regulated by interdomain interactions, with the NT-HBD being a structural or functional modulator of HRI's transformation and catalytic activity.

CHAPTER VI

Summary

Hsp90 is highly conserved and abundant molecular chaperone that facilitates folding of its clients. In so doing, Hsp90 function plays a key role in the regulation of cell physiology. Recent studies indicate that inhibition of Hsp90 function causes destabilization of many cellular proteins that are critically involved in signal transduction pathways. How Hsp90 function may be regulated within a cell is now an active field of investigation.

The studies presented in this thesis focused primarily on the role of Hsp90 in maintaining the function of signal transduction molecules in differentiating C2C12 myoblasts, and the effects of novobiocin on the conformation, function, and client binding affinity of Hsp90 and its co-chaperones. In addition, the molecular mechanism of the regulation of HRI by heme, and NO and CO, gasses, which are known to be second messenger molecules involved in the regulation of signal transduction pathways in the cardiovascular and nervous system, was investigated.

Hsp90 plays a significant role in the regulation of signal transduction pathways during muscle cell differentiation. The inhibition of Hsp90 function by geldanamycin in differentiating C2C12 myoblasts resulted in the depletion of the protein kinases, ErbB2, Akt, and Fyn, and blocked differentiation of myoblasts. These events subsequently led to the induction of apoptosis, as indicated by the cleavage of PARP. These initial studies bring new insights into the essential role for Hsp90 for the proper function of signal

transduction pathways required for myoblast differentiation. Therefore, these studies raise some fundamental questions that need to be answered in the future.

How are the stabilities of Hsp90-dependent kinases differentially regulated in response to Hsp90 inhibition? Both Fyn and c-Src are the members of the Src-family of non-receptor protein tyrosine kinases. They have a similar structure and have overlapping functions. However, the inhibition of Hsp90 function by geldanamycin only affected the stability of Fyn, when previous studies have suggested that c-Src is an Hsp90-dependent client. Therefore, it is important to understand what events have occurred in C2C12 cells that cause the differences in stability between these two kinases. How does the biogenesis of c-Src, a presumptive Hsp90 client, become Hsp90 independent?

How is Hsp90 involved in the regulation of myogenin expression? The expression of myogenin was blocked by geldanamycin treatment. On the other hand, MyoD, whose activity is required for myogenin expression, was observed to interact with Cdc37, but not Hsp90. However, while the interaction of Cdc37 with MyoD was disrupted by geldanamycin, turnover of the protein was not induced by inhibition of Hsp90. Thus, it is of interest to understand the relationship between MyoD function and Hsp90/Cdc37, and why MyoD function is sensitive to geldanamycin. MyoD is known to undergo changes in its phosphorylation state that are thought to regulate its interactions with other myogenic transcription factors. Cdc37 may be acting as a scaffolding protein that is required to dock a protein kinase, which phosphorylates MyoD and modulates its function. Alternatively, geldanamycin may simply block the maturation of an Hsp90-dependent kinase whose function is required for transactivation of MyoD.

How does Hsp90 function to both enhance and dampen the phosphorylation state of a protein client? It was of interest that we found that phosphorylation of Akt on Ser^{473} can be increased by geldanamycin, indicating that Hsp90 functions to balance the phosphorylation state of Akt during C2C12 myoblast differentiation. These results suggest that PP2A is a major regulator of Akt activity. However, the mechanism by which PP2Ac and thus Akt activity is regulated may be more complex, because there are other known and possibly other unidentified Hsp90 clients involved in regulation of Akt activity. Thus, additional work needs to done to study the role of protein phosphatases in regulating Hsp90-dependent signal transduction mechanisms.

What is the true mechanism by which novobiocin inhibits Hsp90's function? Novobiocin has been demonstrated to bind to the C-terminal region of Hsp90, as opposed to the N-terminal region of Hsp90 which binds geldanamycin. Our findings demonstrate that the C-terminal region of Hsp90 is important for the function of Hsp90 and can be the target of action of other drugs. Work remains to be done in defining the drug-binding site within the C-terminal region, which will allow the development of better, higher affinity drugs for treatment of diseases.

What is the true nature of the second heme-binding site in HRI. Our mutagenesis studies on HRI indicate that the interaction between two domains of HRI modulates its activation. Furthermore, the N-terminal heme-binding domain appears to have both negative and positive effects on the regulation of HRI transformation and activity. The question remains whether the second-heme binding site is defined by the interface between the two domains, or whether it is an independent site that is allosterically regulated. However, others have questioned the existence of the site, raising the question

of whether there is a single site that undergoes changes in the coordination state of its bound heme. This hypothesis raises additional question about the relationship between NO binding and regulation of HRI that occurs in response to changes in heme concentrations. Thus, future work is needed to define the structure of HRI and to understand how conformational changes induced by the binding of heme, NO or CO alter HRI's structure between its active and inactive form.

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VITA

Bo-Geon Yun

Candidate for the Degree Of

Doctor of Philosophy

Thesis: STUDIES OF HSP90 FUNCTION ON SIGNAL TRANSDUCTION MOLECULES IN C2C12 CELLS AND EFFECTS OF NOVOBIOCIN ON HSP90 CONFORMATION AND FUNCTION AND MECHANISM OF REGULATION OF HEME-REGULATED eIF2α KINASE BY GAS MOLECULES

Major Field: Biochemistry and Molecular Biology

Biographical:

Personal Data: Born in Seoul, Korea, on December 9, 1970, the son of No-oak Lee and Dong-Shik Yun.

Education: Graduated from Kangseo High School, Seoul, Korea in February 1989; received Bachelor of Science Degree in Biochemistry from Kangwon National University, Kangwon-do, Korea in February 1994; completed requirement for the Doctor of Philosophy Degree with a major in Biochemistry and Molecular Biology at Oklahoma State University in December 2004.

Experience: Half-time Research Assistant, 1997-2004; Quarter-time Research Assistant, Laboratory of Mass Spectrometry, 2002; Department of Biochemistry and Molecular Biology, and Chemistry, Stillwater, OK.

Honors and Awards: Undergraduate scholarship, 1990, Kangwon National University; selected for talk presentation, Midwest Heat Shock Protein Meeting, 2004.

Name: BO-GEON YUN Date of Degree: December, 2004

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: STUDIES OF HSP90 FUNCTION ON SIGNAL TRANSDUCTION MOLECULES IN C2C12 CELLS AND EFFECTS OF NOVOBIOCIN ON HSP90 CONFORMATION AND FUNCTION AND MECHANISM OF REGULATION OF HEME-REGULATED eIF2α KINASE BY GAS MOLECULES

Pages in Study: 149 Candidate for the Degree of Doctor of Philosophy

Major Field: Biochemistry and Molecular Biology

Scope and Method of Study: The purpose of this study was to investigate: the function of Hsp90 on signal transduction pathways required for C2C12 myoblast differentiation; to characterize the effect of the Hsp90-inhibitor novobiocin on the structure and function of Hsp90; and to elucidate the mechanism of regulation of heme-regulated eIF2α kinase (HRI) by the gas molecules, nitric oxide (NO) and carbon monoxide (CO). Hsp90-dependent signaling pathways were examined in differentiating C2C12 myoblasts, using the pharmacological agents geldanamycin, novobiocin, and okadaic acid, pulse-chase labeling, immunoadsorption of protein kinases, and western blotting. The mechanism of inhibition of Hsp90 by novobiocin was analyzed using pull-down assays and western blotting, proteolytic fingerprinting, and protein kinase assays. Regulation of HRI by NO and CO was studied using coupled transcription/translation of site-specific and deletion mutants of HRI in nuclease treated reticulocyte lysate, followed by pull-down and kinase assays to analyze interdomain interactions and the activity of HRI.

Findings and Contributions: Inhibition of Hsp90 function induced apoptosis in differentiating C2C12 myoblasts by depleting ErbB2, Akt(PKB) and Fyn kinases, which modulate signal transduction pathways critical for cell differentiation and survival, and blocking myogenin expression, which is required for transcriptional activation of muscle specific genes. Hsp90 modulated Akt activity via protein phosphatase 2A. Novobiocin bound to the C-terminal region of Hsp90, induced specific conformational changes in Hsp90's structure, altered Hsp90's interaction with its co-chaperone partners, and inhibited the maturation of HRI. These results verify that novobiocin is an Hsp90 inhibitor. The interaction of the N-terminal heme-binding domain (NT-HBD) of HRI with the catalytic domain of HRI was disrupted by NO, but enhanced by CO or heme. A deletion mutant HRI was hyperactivated in heme-deficient lysate, identifying a region within the NT-HBD of HRI that is critical for the regulation of HRI activity and modulating inter-domain interactions.

Adviser's Approval: _____